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ORIGINAL ARTICLE

Observational Study

Histopathological analysis of infiltrating T cell subsets in acute T cell-mediated rejection in the kidney transplant

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Author contributions: Salcido-Ochoa F designed the study, revised all the collected data, analysed data, wrote and revised the paper; Hue SSS, Iqbal J and Loh AHL analysed histopathological data and revised the paper; Peng S and Fan Z collected clinical data and plotted the data; Li RL performed the immunohistochemistry experiments and collected clinical data; Allen Jr JC performed and supervised statistical analysis, and revised the paper.

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Abstract

AIM

To compare the differential immune T cell subset com-

position in patients with acute T cell-mediated rejection in the kidney transplant with subset composition in the absence of rejection, and to explore the association of their respective immune profiles with kidney transplant outcomes.

METHODS

A pilot cross-sectional histopathological analysis of the immune infiltrate was performed using immunohistochemistry in a cohort of 14 patients with acute T cell-mediated rejection in the kidney transplant and 7 kidney transplant patients with no rejection subjected to biopsy to investigate acute kidney transplant dysfunction. All patients were recruited consecutively from 2012 to 2014 at the Singapore General Hospital. Association of the immune infiltrates with kidney transplant outcomes at up to 54 mo of follow up was also explored prospectively.

RESULTS

In a comparison to the absence of rejection, acute T cell-mediated rejection in the kidney transplant was characterised by numerical dominance of cytotoxic T lymphocytes over Foxp3$^+$ regulatory T cells, but did not reach statistical significance owing to the small sample size in our pilot study. There was no obvious difference in absolute numbers of infiltrating cytotoxic T lymphocytes, Foxp3$^+$ regulatory T cells and Th17 cells between the two patient groups when quantified separately. Our exploratory analysis on associations of T cell subset quantifications with kidney transplant outcomes revealed that the degree of Th17 cell infiltration was significantly associated with shorter time to doubling of creatinine and shorter time to transplant loss.

CONCLUSION

Although this was a small pilot study, results support our suspicion that in kidney transplant patients the immune balance in acute T cell-mediated rejection is tilted towards the pro-rejection forces and prompt larger and more sophisticated studies.

Key words: Acute T cell-mediated rejection in the kidney transplant; Banff classification; Cytotoxic T cell; Regulatory T cell; Th17 cell

Core tip: In the clinical setting, acute T cell-mediated rejection in the kidney transplant (ATCMR-KTx) is only confirmed through a kidney transplant biopsy, which is scored according to the Banff classification. The Banff classification is largely based on the estimation of mononuclear cell infiltration instead of the identification and quantification of the actual T cell subsets recruited to mediate rejection. Therefore, a more detailed analysis of the inflammatory infiltrate of ATCMR-KTx is likely to enhance the diagnostic accuracy of the Banff classification. In our analyses, ATCMR-KTx appeared to be characterised by a numerical dominance of cytotoxic T lymphocytes over regulatory T cells in comparison to the absence of acute rejection. We also found an association of the numbers of infiltrating Th17 cells with kidney transplant outcomes. Although this is a small pilot study, it further supports our suspicion that the immune balance in ATCMR-KTx is tilted towards the pro-rejection forces.

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INTRODUCTION

Acute T cell-mediated rejection in the kidney transplant (ATCMR-KTx) is a common encounter in kidney transplantation. It can perpetuate itself as chronic T cell-mediated rejection or transform into antibody-mediated rejection, which progressively can destroy the renal parenchyma, leading to reduction of kidney transplant survival with potential transplant loss and the return to dialysis[1,2]. Therefore, adequate maintenance immunosuppression to prevent the occurrence of ATCMR-KTx, prompt and accurate identification, and early initiation of anti-rejection therapy are needed to minimise patient's complications and to improve long-term kidney transplant outcomes.

In the current state of the art, confirmation of ATCMR-KTx is based on scoring kidney transplant histopathological changes using the Banff classification[3]. Despite being the gold standard, there are a few limitations. The Banff classification relies on a semi-quantitative estimation of the infiltrating mononuclear cells. This approach, however, does not distinguish the actual cellular program that is operating within the transplant tissue. We believe that identification of the actual T cell subsets infiltrating the kidney transplant provides better insight into the immunologic events in ATMCR-KTx. In other words, a more detailed analysis of the inflammatory infiltrate of kidney transplant biopsies undergoing ATCMR-KTx is expected to reflect more accurately the status of alloactivation within the kidney transplant and to lead to a better understanding of the immunopathogenesis of ATCMR-KTx. Similarly, this information could be used in the future to improve the accuracy and the predictive value of the Banff classification in kidney transplantation.

The immunologically-mediated damage of ATCMR-KTx is mediated and executed by different subtypes of effector T cells, including cytotoxic T lymphocytes (CTL), T helper (Th) 17 cells and Th1 cells, as well as natural killer cells and monocytes. In addition, Foxp3$^+$ regulatory T cells (Treg cells) are known to migrate also to the transplant tissue to modulate the inflammatory response[4-9].

CTL are central effectors of alloimmune damage to the parenchymal cells of the kidney transplant[10,11]. Therefore, the detection of their cytotoxic products inside the kidney transplant is commonly used as a surrogate of their presence and their allotoxicity. To highlight a few examples, at the molecular level intra-graft detection of granzyme B mRNA has been shown to be able to differentiate ATCMR-KTx from the absence of rejection[12,13]. Concomitant detection of both granzyme B and perforin mRNA[14,15], or of both granzyme B and CD178 mRNA[13,16] have also been shown to identify ATCMR-KTx with higher accuracy. It has also been reported that the detection of granulysin mRNA, another CTL product, helped to differentiate patients with ATCMR-KTx from those with no rejection in their biopsies[17]. A similar result has also been observed at the protein level by immunohistochemical detection of granzyme B and perforin expression[10]. Although the outcome of kidney transplantation after an episode of ATCMR-KTx is difficult to predict, there are some indications that the detection of markers of CTL in the kidney transplant may offer some value. One study demonstrated that a higher degree of granzyme B^+ cell infiltration in the allograft was associated with poorer allograft survival[18], and another study showed that the intra-graft expression of granzyme B was associated with the severity of the rejection process[10]. Likewise, the expression of CD178[19] or the co-expression of both CD178 and granzyme B[13] conferred poorer prognosis to patients suffering from ATCMR-KTx. Despite the aforementioned findings, it has been suggested that expression of granzyme B by itself may have limited clinical predictive value[19].

Th17 cells are another type of effector T cells involved in alloimmunity and in biopsies are usually identified by the detection of IL-17. It has been reported that the magnitude of Th17 cell infiltration over Treg cell infiltration correlated with kidney transplant function, the degree of interstitial inflammation and tubular atrophy, the refractoriness to treatment and the recurrence of ATCMR-KTx[20-22].

Despite the belief that Th1 cells are believed to be crucial mediators of the rejection process, the detection of interferon-gamma, as a surrogate marker for their presence, was no better than the detection of cytotoxic molecules for the diagnosis of pure ATCMR-KTx[13]. In addition, intra-graft expression of T-bet, also a surrogate marker for Th1 cells, was not able to distinguish ATCMR-KTx from the absence of rejection. In this respect, the role of Th2 cells in the rejection process appears to be less dramatic and less understood; and the identification of Th2 cells through the detection of intra-graft IL-4 mRNA was also not useful for the diagnosis of ACTMR-KTx[13].

Although several reports have implicated $Foxp3^+$ Treg cells in alloregulation and transplantation tolerance in animal models[8,23] and in humans[24], the detection of $Foxp3^+$ Treg cells to aid in the diagnosis of ATCMR in the kidney transplant and their clinical significance has been beset with controversy[25]. Some authors have published that higher infiltration by $Foxp3^+$ Treg cells appeared to associate with more favourable transplant outcomes in patients with ATCMR-KTx[26] and in patients with subclinical rejection found in protocol biopsies[27,28], in comparison to those cases of much lower infiltration by $Foxp3^+$ Treg cells. Likewise, patients with ATCMR-KTx having higher expression of Foxp3 mRNA were more likely to respond to therapy that those with lower levels[20]. However, other studies reported were not very supportive of the detection of Treg cells in ATCMR-KTx. The detection of intra-graft Foxp3 mRNA, as a surrogate marker for $Foxp3^+$ Treg cells, was not associated with the diagnosis ATCMR-KTx in one study[12]. In addition, no association was found in another study of ATCMR-KTx between the detection of $Foxp3^+$ T cells by immunofluorescence and kidney transplant outcomes[29].

We have hypothesised that the balance between effector and $Foxp3^+$ Treg cells could play a role in determining the occurrence and severity of ATCMR-KTx, as well as predicting the potential outcome of the kidney transplant[25]. However, as discussed previously, the clinical significance of the immune infiltrate in ATCMR-KTx or its balance is controversial. Therefore, in this study performed in a cohort of Asian patients, we aimed to identify and quantify the main T cell subsets infiltrating the kidney transplant undergoing ATCMR and to compare with that in the absence of rejection. We use immunohistochemistry as our detection technology as it is inexpensive, easily reproducible and accessible to many laboratories. Based on the literature presented above, we focused our immunodetection on the most promising markers, *i.e.*, granzyme B and IL-17 (representing CTL and Th17 cells, respectively) and Foxp3 (representing $Foxp3^+$ Treg cells). To assess their immune balance, we arbitrarily measured their numerical ratios within the immune infiltrate found in both kidney transplant patients with ATCMR-KTx and with no rejection. Then, we explored the association of the numbers of these subsets and their ratios with kidney transplant outcomes up to fifty-four months of clinical follow up. We focused our outcome analysis on the risk of subsequent rejection episodes, deterioration of kidney transplant function and immunologically-mediated transplant loss.

MATERIALS AND METHODS

Study design

Cross-sectional immunohistochemical analysis performed in formalin-fixed paraffin-embedded tissue collected in a consecutive cohort of 21 kidney transplant patients that were subjected to kidney transplant biopsy for the investigation of acute kidney transplant dysfunction at any time post-transplantation. Patients satisfying our inclusion and exclusion criteria were subdivided post-hoc into two groups: (1) ATCMR-KTx; and (2) no rejection. All patients were recruited

between 1 January 2012 to 1 January 2014 at the Singapore General Hospital (SGH), the largest tertiary care and academic centre in Singapore; and followed for kidney transplant outcomes up to fifty-four months from the time of transplant biopsy.

Patient characteristics

Inclusion criteria: Adult kidney transplant patients (aged 21-80 years) who were of low immunological risk (ABO-compatible, lack of donor-specific antibodies, negative cross-match, no history of antibody-mediated rejection); who had acute kidney transplant dysfunction due to: (1) ATCMR-KTx (category 4 of the Banff 2009 classification); or (2) found with absence of rejection in the biopsy (category 1 of the Banff 2009 classification, or category 6 of the Banff 2009 classification of no inflammatory or infective nature, *i.e*., with no BK virus nephropathy, other infections affecting the transplant, glomerulonephritis or interstitial nephritis).

Exclusion criteria: Human immunodeficiency virus infection, history of haematological malignancies, children, pregnant women, poor cognitive capacity, prisoners and the inability to understand the research protocol and give consent. Patients whose biopsies showed borderline rejection (category 3 of the Banff 2009 classification) or antibody-mediated rejection (category 2 of the Banff 2009 classification) were also excluded from the analysis. Biopsies in the non-rejection group were revised according to the Banff 2013 update before the final analysis, to ensure they still satisfy the non-rejection group criteria according to the Banff 2013 update.

Clinical data

Baseline demographic and clinical characteristics as well as clinical outcomes were retrieved from clinical hard-copy case notes and our electronic medical records. Use and type of immunosuppressants prescribed were also recorded.

Routine laboratory investigations

Serum creatinine and urine protein to creatinine ratio (or total urinary protein in a 24-h collection) were measured. Calculated estimated glomerular filtration rate (eGFR) was obtained through the "modification of diet in renal disease" equation. All laboratory parameters were retrieved prospectively from electronic medical records from the time of kidney transplant biopsy and at 3, 6, 12, 18, 24, 30, 36, 42, 48 and 54 mo of follow up post-biopsy. All laboratory investigations were conducted at the SGH's clinical laboratory, which is accredited by the College of American Pathologists.

T cell subset detection in kidney transplant biopsies by immunohistochemistry

Immunohistochemistry for detection of T cell subsets in kidney transplant tissue biopsies was performed in both the Renal Laboratory and the Pathology Laboratory at the SGH. In brief, slides prepared from formalin-fixed paraffin-embedded kidney tissue specimens were stained with monoclonal antibodies conjugated with either horseradish peroxidase or alkaline phosphatase and directed against different phenotypic markers, including CD4, CD8, CD19, IL-17, granzyme B and Foxp3. The binding of the different antibodies onto the kidney tissue samples was revealed using the respective chromogenic substrates for those enzymes. Isotype-matched antibodies were used as negative controls. Tonsil tissue served as positive control. Staining was visualized and quantified directly by light microscopy and adjusted to biopsy tubulo-interstitial area (vessels and glomeruli excluded) measured by Olympus CellSens software. Percentage of infiltration of $CD4^+$, $CD8^+$ and $CD19^+$ cells, as well as the number of Foxp3-, IL-17- or granzyme B-expressing cells per square millimetre of kidney tubulo-interstitial area in the biopsy (cell density) was reported. The ratios between the cell densities of granzyme B- and IL-17-expressing cells over Foxp3-expressing cells were calculated.

Statistical analysis

Sample size: As this was an exploratory study on consecutively recruited patients, sample size was not calculated a priori.

To determine whether tissue-infiltrating T cell profiles differ between kidney transplant patients with: (1) biopsy-proven ATCMR-KTx; and (2) no rejection, median cell densities of tissue-infiltrating: (1) granzyme B^+ CTL; (2) IL-17^+ Th17 cells; (3) $Foxp3^+$ Treg cells were compared between these two groups of patients. In addition, ratios of the cell densities of tissue-infiltrating; (4) granzyme B^+ CTL over $Foxp3^+$ Treg cells; and of (5) IL-17^+ Th17 cells over $Foxp3^+$ Treg cells were compared between kidney transplant patients with: (1) biopsy-proven ATCMR-KTx; and (2) no rejection. Medians were compared using the Wilcoxon rank-sum test. Spearman correlation was used to assess strength of association of densities and ratios of infiltrating immune cells with different kidney transplant outcomes, including: (1) changes in serum creatinine; (2) eGFR; and (3) proteinuria. Longitudinal analysis of variance was used to display and compare changes in these same outcome variables between the two groups of patients over the follow up period. The analysis was performed on log-transformed values in order to achieve normality of residuals. The log-rank test was used to compare time-to-event curves between the biopsy-proven ATCMR-KTx and the no-rejection groups for the following outcomes: (1) time to any rejection (a composite outcome including borderline rejection, ATCMR-KTx or antibody-mediated rejection occurring post-biopsy during the follow up period); (2) time to doubling of creatinine post-biopsy; and (3) time to confirmed or suspected immune-mediated transplant loss. The date for re-initiation of dialysis was taken as the date of transplant

Table 1 Baseline clinical and demographic characteristics of the kidney transplant patients

Characteristic	n[3]	No rejection	n[4]	ATCMR	*P* value
Age (yr)[1]	7	60.8	14	44.9	0.0101
Male sex (%)	7	57.14	14	71.43	0.6384
Race Chinese (%)	7	86.71	14	57.14	0.3371
Dialysis vintage (yr)[1]	7	2.08	14	5.015	0.6888
Transplant vintage (yr)[1]	7	13.75	14	3.935	0.0031
Deceased donor (%)	6	66.67	13	53.85	> 0.9999
Delayed graft function (%)	6	33.33	12	41.67	> 0.9999
Cold ischaemia time (h)	5	3	9	10	0.6973
Total HLA mismatch (#)[1]	6	3	11	3	0.9973
Very high immune risk (%)[2]	6	16.67	11	43.45	0.3334
% Panel of reactive antibodies[1]	3	8	9	0	0.2318
History of ATCMR (%)	7	14.29	14	50	0.1736
Re-transplant (%)	7	0	14	7.14	> 0.9999
GFR at biopsy (mL/min per 1.73 m^2)[1]	7	41.2	14	17.95	0.0767
Proteinuria at biopsy (g/d)[1]	7	3.5	14	1.23	0.2028
t score[1]	7	0	14	2	0.0116
I score[1]	7	1	14	2	0.0007
v score[1]	7	0	14	0	0.1196
Tacrolimus use at biopsy (%)	7	0	14	50	0.0468
Ciclosporin use at biopsy (%)	7	100	14	35.71	0.0071
MTORI use at biopsy (%)	7	0	14	14.29	0.5333
Steroids use at biopsy (%)	7	100	14	100	> 0.9999
Mycophenolate use at biopsy (%)	7	57.14	14	85.71	0.28
Azathioprine use at biopsy (%)	7	28.57	14	0	0.10
Anti-CD25 induction (%)	5	0	12	41.67	0.2445
Prior thymoglobulin use (%)	7	14.29	14	14.29	> 0.9999

[1]Results reported as median values; [2]According to United Kingdom Fuggle's classification based on HLA-DRB1 and HLA-B mismatches[30]; [3]Indicates the number of patients with available data in the non-rejection group; [4]Indicates the number of patients with available data in the ATCMR-KTx group. ATCMR: Acute T cell-mediated rejection; GFR: Glomerular filtration rate; HLA: Human leukocyte antigen; MTORI: Mammalian target of rapamycin inhibitor.

loss. Cox regression analysis was used to investigate the effect of the cell densities of the tissue infiltrating T cells and their ratios and other clinical parameters (potential confounders taken from Table 1) on different kidney transplant outcomes including: (1) time to any rejection post-biopsy; (2) time to doubling of creatinine post-biopsy; and (3) time to confirmed or suspected immune-mediated transplant loss. All analyses were performed using SAS V9.4 software (SAS Inc., Cary NC, United States).

RESULTS

Table 1 shows the main clinical and demographic characteristics of the 21 recruited kidney transplant patients that were subjected to transplant biopsy for the investigation of acute kidney transplant dysfunction (14 with ATCMR-KTx and 7 with no rejection, inflammation nor infection found in their biopsy). Overall, in the ATCMR-KTx group the need for transplant biopsy occurred earlier post-transplantation than for the non-rejection group and had worse kidney function at presentation. History of previous rejection episodes occurred preferentially in this group too. They also had slightly higher rate of delayed graft function and longer cold-ischaemia than the non-rejection group. The HLA mismatches and the immune risk according to Fuggle's classification[30] was similar in both groups. In our patient cohort, all the non-rejection patients were taking ciclosporin as maintenance immunosuppression at the time of the biopsy, while half of the patients in the ATCMR-KTx group were on tacrolimus. The acute rejection scores (*t*, *i* and *v*) of the Banff classification were higher in the ATCMR-Tx group, as expected. Tables 2 and 3 provide the detailed clinical and demographic characteristics of each recruited patient, as well as their particular immune variables and main kidney transplant outcomes.

Comparable infiltration of CTL, Treg cells and Th17 cells in ATCMR-KTx and the absence of rejection

Figure 1 shows a representative panel of the immunohostochemical analysis of T cell subsets in a patient with ATCMR-KTx. The percentage of $CD4^+$ and $CD8^+$ cell infiltration was higher in patients with ATCMR-KTx (Figure 2A and B), and there was no significant difference in B cell infiltration (Figure 2C) in comparison to patients with no rejection. The infiltration of granzyme B^+ cells (surrogates for CTL), $Foxp3^+$ cells (surrogates for Treg cells) and IL-17^+ cells (surrogates for Th17 cells), quantified as cell densities (number of cells per mm^2 of tubulo-interstitial biopsy area), were not statistically different between the two patient groups (Figure 2D-F). Nonetheless, a few ATCMR-KTx patients

Table 2 Baseline clinical and demographic characteristics of the kidney transplant patients

Patient	Group	Age	Sex	Race	Dialysis vintage (yr)	Tx vintage (yr)	Donor type	DGF	CIT (h)	HLA MM (#)	Immune risk	PRA (%)	ATCMR Hx	Re-Tx	Anti-CD25 induction	ATG use	Immuno-suppression at Bx
1	ATCMR	49.9	M	Ma	0.36	14.26	Living	No	0	0	Low	UNK	Yes	No	No	No	CsA + MPA
2	ATCMR	32.1	F	Ch	0.38	0.17	Living	No	UNK	1	Very high	20	Yes	No	Yes	No	MTORI + MPA
3	ATCMR	25.7	M	Ma	1.21	6.80	UNK	UNK	UNK	UNK	UNK	UNK	Yes	No	UNK	No	Tac + MPA
4	ATCMR	36.7	M	Ma	9.48	0.45	Deceased	Yes	10	3	High	7	Yes	No	Yes	No	Tac + MPA
5	ATCMR	59.4	M	Ch	8.68	3.90	Deceased	No	9	4	Very high	7	Yes	No	Yes	No	CsA + MPA
6	ATCMR	46.0	F	Ch	1.20	2.34	Living	No	0	1	Moderate	0	No	No	Yes	No	CsA + MPA
7	ATCMR	40.6	M	Ch	0.31	1.03	Living	No	UNK	UNK	UNK	UNK	No	No	No	Yes	Tac + MPA
8	ATCMR	44.1	M	Ch	9.52	8.09	Deceased	Yes	23	2	High	0	Yes	Yes	No	Yes	Tac
9	ATCMR	56.9	M	Ch	7.98	13.8	Deceased	Yes	UNK	3	High	UNK	No	No	No	No	CsA + MPA
10	ATCMR	45.6	M	Ch	1.08	1.26	Living	UNK	UNK	UNK	UNK	UNK	No	No	UNK	No	Tac + MPA
11	ATCMR	51.5	M	In	8.29	5.34	Deceased	No	19	4	Very high	0	Yes	No	No	No	Tac + MPA
12	ATCMR	57.4	F	Ma	9.31	2.38	Deceased	Yes	15	3	High	0	No	No	Yes	No	Tac + MPA
13	ATCMR	43.6	M	Ch	8.87	3.97	Deceased	Yes	14	5	Very high	3	No	No	No	No	CsA
14	ATCMR	30.6	F	Ma	2.05	11.86	Living	No	5	2	Very high	0	No	No	No	No	MTORI + MPA
15	NR	51.9	M	Ch	0.65	13.75	Living	No	0	4	High	UNK	No	No	No	Yes	CsA + MPA
16	NR	65.1	M	Ch	2.08	18.21	Living	No	UNK	0	Low	UNK	No	No	UNK	No	CsA + MPA
17	NR	61.9	M	Ch	5.88	10.31	Deceased	No	3	3	High	8	No	No	No	No	CsA
18	NR	64.4	F	Ch	2.03	18.36	Deceased	No	16	1	Moderate	33	Yes	No	No	No	CsA + AZA
19	NR	51.0	M	Ch	1.44	11.34	UNK	UNK	UNK	UNK	UNK	UNK	No	No	UNK	No	CsA + MPA
20	NR	43.6	F	Ch	3.24	19.81	Deceased	Yes	1.2	3	High	UNK	No	No	No	No	CsA + AZA
21	NR	60.8	F	Ma	4.42	8.86	Deceased	Yes	18	4	Very high	0	No	No	No	No	CsA + MPA

ATCMR: Acute T cell-mediated rejection; ATG: Anti-thymocyte globulin; AZA: Azathioprine; Bx: Biopsy; Ch: Chinese; CIT: Cold ischaemia time; CsA: Ciclosporin; DGF: Delayed graft function; F: Female; HLA: Human leukocyte antigen; Hx: History; In: Indian; M: Male; Ma: Malay; MM: Mismatch; MPA: Mycophenolic acid analogue; MTORI: Mammalian target of rapamycin inhibitor; NR: No rejection; PRA: Panel of reactive antibodies; Tac: Tacrolimus; Tx: Transplant; UNK: Data unknown.

Table 3 Immune infiltrate characteristics and outcomes of the kidney transplant patients

Patient	Group	t	i	v	CD4 (%)	CD8 (%)	CD19 (%)	Granzyme B (cells/mm^2)	IL-17 (cells/mm^2)	Foxp3 (cells/mm^2)	CTL/Treg ratio	Th17/Treg ratio	GFR at Bx	GFR last follow-up	Protei-nuria at Bx	Protei-nuria last follow-up	Time to any rejection (d)	Time to doubling of creatinine (d)	Time to Tx loss (d)	Total follow-up (d)
1	ATCMR	1	3	1	35	25	15	68	5	35	2	0.1	18.5	4.7	4.28	UNK	NA	38	116	116
2	ATCMR	3	2	0	60	60	10	346	2	149	2.3	0	33.6	67.2	0.51	0.16	28	NA	NA	1643
3	ATCMR	2	2	1	30	35	30	31	15	73	0.4	0.2	48.1	30.9	0	UNK	92	NA	NA	1623
4	ATCMR	2	2	0	30	25	30	55	17	56	1	0.3	15.2	9.5	0.41	1.71	NA	NA	513	513
5	ATCMR	3	2	1	85	80	25	544	19	311	1.8	0.1	11.2	15	1.08	1.61	NA	NA	645	645
6	ATCMR	2	1	0	30	15	10	26	52	3	8.8	17.9	30.1	6.7	0.39	UNK	1037	941	1176	1176
7	ATCMR	0	1	1	10	20	10	42	4	6	6.6	0.6	49.8	70.3	0.32	0.09	NA	NA	NA	1327
8	ATCMR	0	2	1	5	10	0	13	0	8	1.5	0	16.9	6.2	2.43	6.66	164	164	164	164
9	ATCMR	2	2	0	10	5	10	4	43	1	4.4	47.4	17.4	14.3	2.34	UNK	NA	NA	759	759
10	ATCMR	1	2	0	35	50	15	81	20	17	4.7	1.2	25.8	8.6	1.53	2.46	404	911	933	933
11	ATCMR	1	1	1	10	5	5	9	22	2	4.7	11.5	112.1	44.7	2.07	0.07	NA	520	NA	950
12	ATCMR	1	2	0	20	15	10	18	5	4	4.8	1.3	16.4	15.1	0.58	UNK	NA	NA	NA	917
13	ATCMR	3	2	0	80	70	20	322	32	35	9.3	0.9	9.2	9.2	1.39	1.39	NA	NA	NA	1
14	ATCMR	2	2	0	20	10	10	38	62	10	3.9	6.3	15.2	8.2	6.09	UNK	NA	NA	NA	913
15	NR	0	1	0	20	15	10	36	55	34	1	1.6	21.1	8.8	6.77	UNK	NA	598	862	862
16	NR	0	1	0	5	10	10	1	5	2	0.5	2.5	43.4	35.8	0.13	1.2	NA	NA	NA	1507
17	NR	0	1	0	5	15	10	5	15	4	1.3	3.8	41.2	63.5	0.39	0.57	NA	NA	NA	1306
18	NR	1	1	0	35	30	30	92	16	32	2.8	0.5	56	9.8	2.4	2.24	NA	974	1118	1118
19	NR	0	1	0	0	5	0	21	2	8	2.7	0.2	64.6	53.2	3.62	7.57	1168	NA	NA	1173
20	NR	1	0	0	20	15	10	25	73	15	1.7	5	28.4	7.1	3.5	UNK	NA	188	520	520
21	NR	1	1	0	25	15	10	81	81	10	8.1	8.1	20.9	12.4	10.59	7.46	NA	141	163	163

ATCMR: Acute T cell-mediated rejection; Bx: Biopsy; GFR: Glomerular filtration rate; HLA: Human leukocyte antigen; i: i score; NA: Not applicable; NR: No rejection; t: t score; Tx: Transplant; UNK: Data unknown; v: v score.

had higher infiltration by granzyme B$^+$ and Foxp3$^+$ cells and are referred subsequently in the text as 'high infiltration outliers'.

Infiltrating CTL appear to numerically overwhelm Treg cells in ATCMR-KTx

As an arbitrary measurement of immune balance within

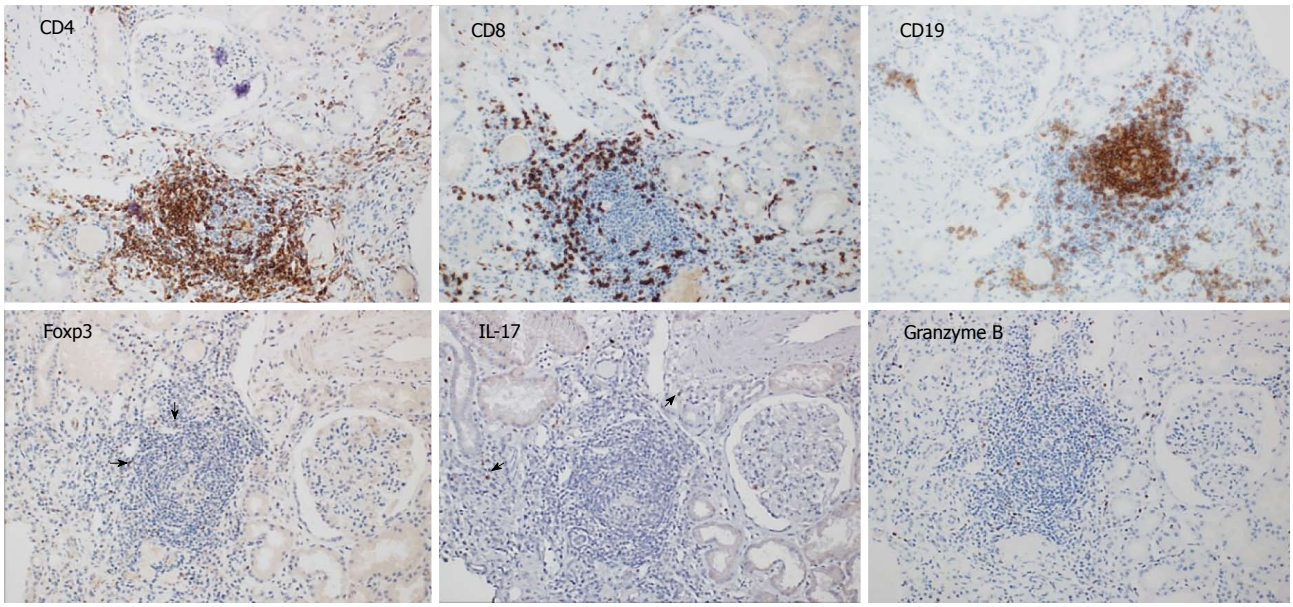

Figure 1 Representative T cell subsets infiltrating a kidney transplant undergoing acute T cell-mediated rejection using antibodies to CD4, CD8, CD19, Foxp3, IL-17 and granzyme B as labeled on the pictures (the arrows indicate positive cells). All pictures derived from the same region cut at consecutive levels (immunohistochemistry staining, magnification × 200).

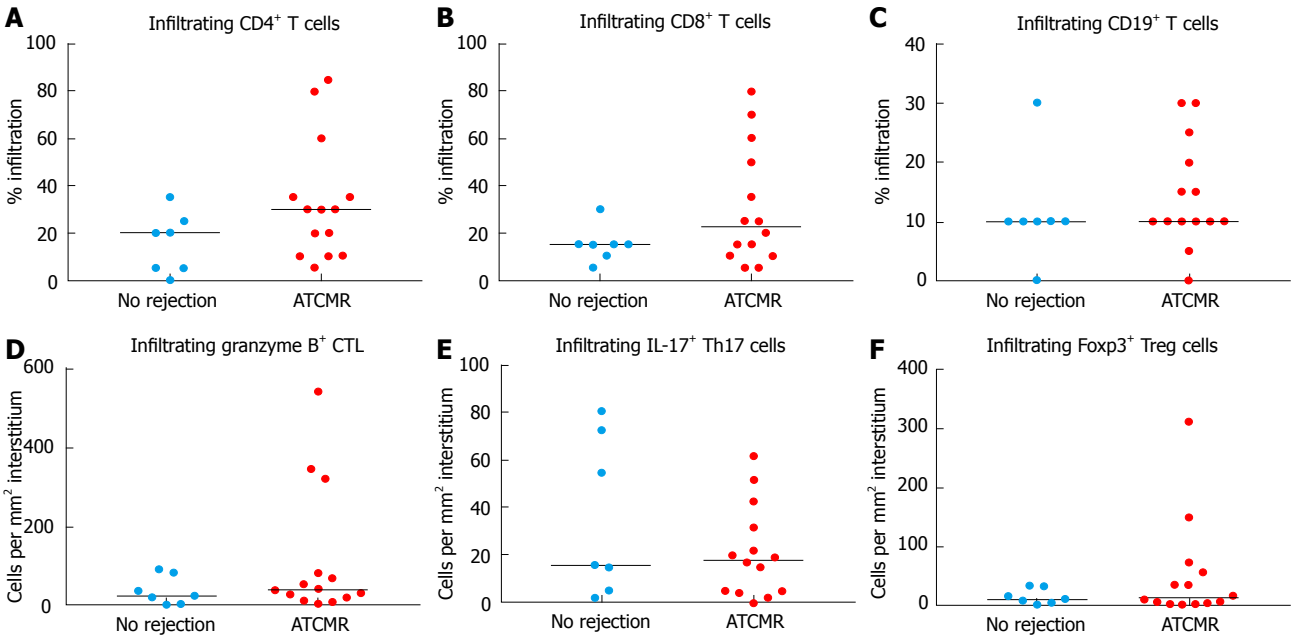

Figure 2 T cell subsets infiltrating kidney tissue, including %CD4$^+$ cells (A), %CD8$^+$ cells (B), %CD19$^+$ cells (C), granzyme B$^+$ cells/mm^2 (D), IL-17$^+$ cells/mm^2 (E) and Foxp3$^+$ cells/mm^2 (F) (all detected by immunohistochemistry) are compared between patients with acute T cell-mediated rejection in the kidney transplant (n = 14) and patients with no rejection (n = 7). The horizontal lines indicate the median values. Wilcoxon rank-sum test P values for all comparisons were statistically non-significant. ATCMR: Acute T cell-mediated rejection; CTL: Cytotoxic T lymphocyte.

the kidney transplant, the granzyme B$^+$ cell to Foxp3$^+$ cell density ratio was found to be higher in patients with ATCMR-KTx than for patients in which rejection was not observed (Figure 3A). However, the ratio of infiltrating IL-17-producing cells over Foxp3$^+$ cells was not much different in patients with ATCMR-KTx than in patients not experiencing rejection (Figure 3B). Given our small sample size, these comparisons did not achieve statistical significance. However, once more there were a few "high infiltration outliers" for the ratio of infiltrating Th17 cells over Foxp3$^+$ Treg cells.

Th17 cell infiltration in ATCMR-KTx associates with worse kidney transplant function

The numbers of infiltrating Th17 cells in the ATCMR-KTx patients were significantly positively correlated with serum creatinine levels and proteinuria, and negatively correlated with eGFR at different time points during follow up. The numbers of infiltrating Th17 cells and the ratio of Th17 cells over Foxp3$^+$ Treg cells in the non-rejection patients were

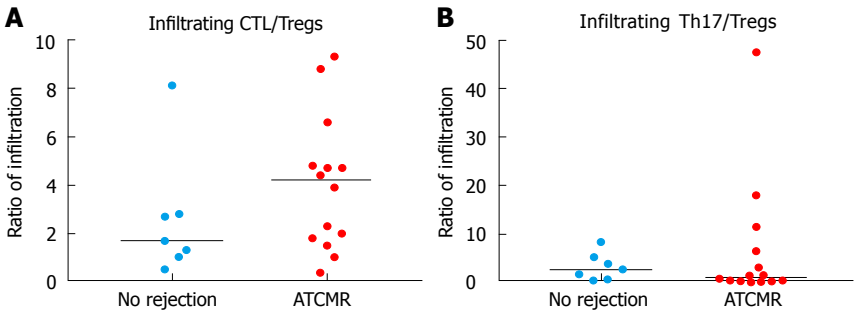

Figure 3 The ratios of (A) infiltrating granzyme B$^+$ cells (CTL) over Foxp3+ cells (Tregs) and of (B) of infiltrating IL-17$^+$ cells (Th17) over Foxp3$^+$ cells (Tregs) are compared between patients with acute T cell-mediated rejection in the kidney transplant (*n* = 14) and patients with no rejection (*n* = 7). All cell types were detected by immunohistochemistry. The horizontal lines indicate the median values. Wilcoxon rank-sum test p values for both comparisons were statistically non-significant. ATCMR: Acute T cell-mediated rejection; CTL: Cytotoxic T lymphocyte.

Table 4 Correlation (R) between numbers and ratios of infiltrating immune cells and kidney transplant outcomes

Group	Immune parameter	*vs*	Outcome	R	*P* value
No rejection	Infiltrating Th17 cells		Creatinine t3	0.9429	0.0167
No rejection	Infiltrating Th17 cells		GFR t0	-0.8571	0.0238
No rejection	Infiltrating Th17/Tregs		GFR t0	-0.7857	0.048
No rejection	Infiltrating Th17 cells		GFR t3	-0.9429	0.0167
No rejection	Infiltrating Th17/Tregs		GFR t3	-0.9429	0.0167
No rejection	Infiltrating Th17 cells		GFR t6	-0.8929	0.0123
ATCMR-KTx	Infiltrating CTL/Tregs		Creatinine t3	-0.6694	0.0145
ATCMR-KTx	Infiltrating Th17 cells		Creatinine t24	0.6485	0.049
ATCMR-KTx	Infiltrating Th17 cells		Creatinine t30	0.7619	0.0368
ATCMR-KTx	Infiltrating Th17 cells		GFR t30	-0.8333	0.0154
ATCMR-KTx	Infiltrating Th17 cells		Proteinuria t12	0.8095	0.0218

ATCMR-KTx: Acute T cell-mediated rejection in the kidney transplant; GFR: Glomerular filtration rate.

significantly positively correlated with serum creatinine levels and negatively correlated with eGFR at different time points during follow up. Correlation estimates and *P* values of the statistically significant associations are shown in Table 4. The numbers of infiltrating CTL and infiltrating Foxp3$^+$ Treg cells were not significantly associated with any of the clinical outcomes tested including changes in serum creatinine, eGFR or proteinuria. However, a significant negative correlation of the ratio of infiltrating CTL over Foxp3$^+$ Tregs with creatinine at 3 mo was observed in ATCMR-KTx patients. Figure 4 shows the dynamic changes in serum creatinine, eGFR and proteinuria throughout the follow up period. The ATCMR-KTx group had overall worse kidney transplant function during follow up than the non-rejection group, while the non-rejection group had overall higher levels of proteinuria. There was no more rapid deterioration in the ATCMR-KTx patients in comparison to the non-rejection patients, as indicated by the absence of statistically significant differences between respective mean values for changes in serum creatinine, eGFR and proteinuria. The time-to-event plots for any rejection post-biopsy (borderline, ATCMR-KTx or antibody-mediated rejection), time to doubling of creatinine post-biopsy, and time to confirmed or suspected immune-mediated transplant loss are found in Figure 5. Table 5 contains the respective median times to event. The comparisons of the time-to-event curves by log rank test were not statistically significant. The effect of the cell densities of the infiltrating immune cells and their ratios, as well as the effect of clinical parameters suspected to influence kidney transplant outcomes (*i.e.*, the potential confounders for kidney transplant outcomes taken from Table 1) were tested using cox regression model. Their respective hazard ratios and 95%CI are shown in Table 6. In the univariate analysis, younger age was associated significantly with shorter time to any rejection. In addition, the number of infiltrating Th17 cells and the degree of proteinuria at biopsy were significantly associated with shorter time to doubling of creatinine. The number of infiltrating Th17 cells, serum creatinine at biopsy and the occurrence of delayed graft function were significantly associated with shorter time to transplant loss. Multivariate analysis was not performed in consequence of the small sample size.

Finally, for ATCMR-KTx patients, Kaplan-Meier time-to-event curves for kidney transplant outcomes corresponding to "high infiltration outlier" patients were compared to outcomes for "non-outlier" patients relative to: (1) number of infiltrating CTL; (2) number of infiltrating Foxp3$^+$ Treg cells; and (3) ratio of Th17 cell to Foxp3$^+$ Treg cell. Owing to the small sample sizes, median time-to-event was not obtainable for any outcome, and differences between "outlier" and "non-outlier" survival curves were non-significant for all three outcome variables (data not shown).

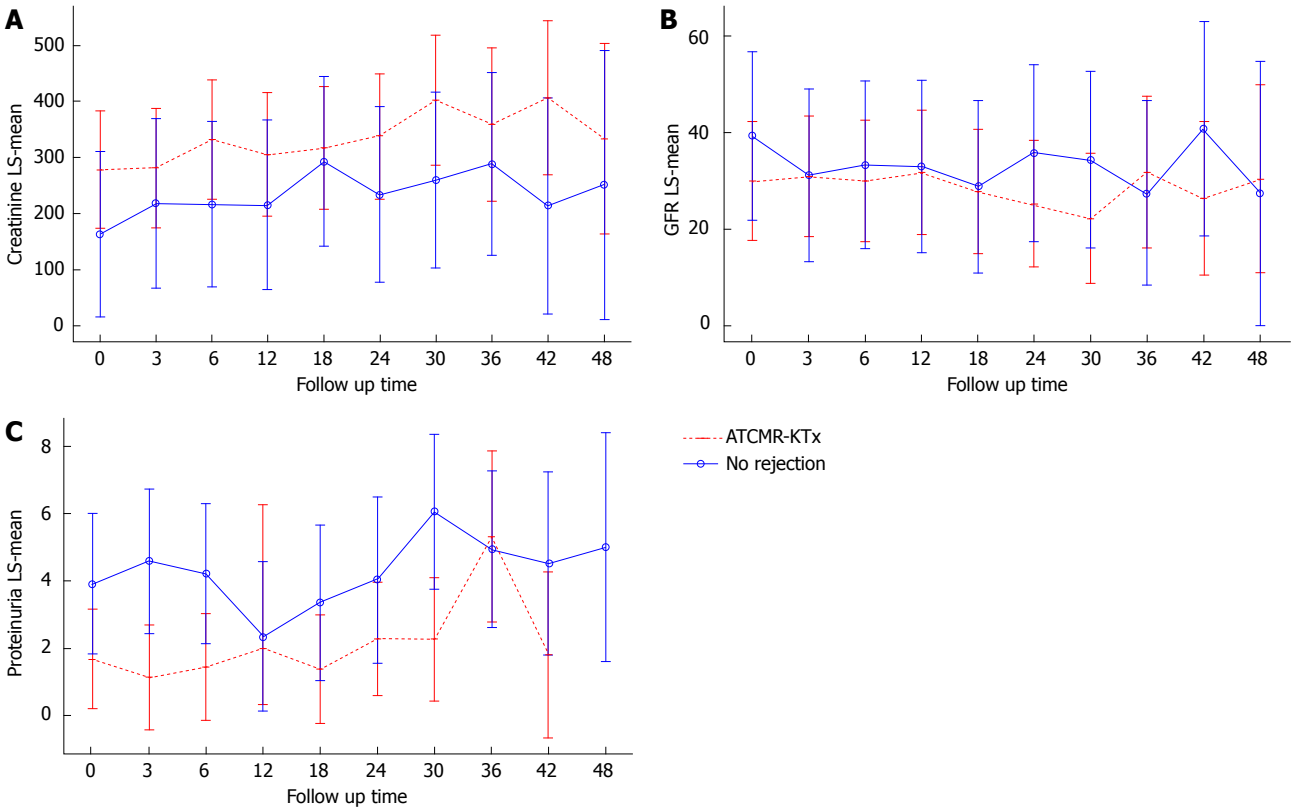

Figure 4 Longitudinal analysis comparing the dynamic changes in serum creatinine (A), glomerular filtration rate (B) and proteinuria (C) throughout the follow up period in the acute T cell-mediated rejection in the kidney transplant (red non-continuous line) and non-rejection (blue continuous line) groups. The comparisons between overall mean values and mean values at follow-up times were statistically non- significant. Upper and lower limits for 95%CIs at the different time points are indicated. ATCMR-KTx: Acute T cell-mediated rejection in the kidney transplant; GFR: Glomerular filtration rate.

Table 5 Comparison of time to transplant outcomes in the kidney transplant patients

Outcomes	Group	Median time-to-event	*P* values
Any rejection	ATCMR	1037	0.0941
	No rejection	Undefined[1]	
Doubling of creatinine	ATCMR	941	0.7452
	No rejection	974	
Transplant loss	ATCMR	1176	0.956
	No rejection	1118	

[1]Median time-to-event was not obtainable (see Figure 4A). ATCMR: Acute T cell-mediated rejection.

DISCUSSION

In this study, our main aim was to determine whether the T cell subset composition in ATCMR-KTx differed qualitatively or quantitatively from that in the absence of rejection. Our main focus was on the numbers and respective ratios of CTL, Th17 cells and Foxp3$^+$ Treg cells, thought to be the most relevant subsets implicated in ATCMR-KTx, according the previously presented literature. ATCMR-KTx appeared to be characterised by a numerical dominance of CTL over Foxp3$^+$ Treg cells in comparison to the absence of acute rejection, suggesting that the immune balance in ATCMR-KTx appears to be tilted to the pro-rejection forces; which might be overwhelming the regulatory forces. This finding is congruent with the literature reports, where the presence of CTL infiltrating the kidney transplant undergoing ATCMR is a characteristic to differentiate ATCMR-KTx from the absence of rejection[10,12,13]; and with the published observation that a lower infiltration by Foxp3$^+$ Treg cells in the kidney transplant undergoing ATCMR was associated with poorer transplant outcomes[26], or with poorer responsiveness to anti-rejection therapy[20].

Our analysis of kidney transplant outcomes revealed that the number of infiltrating Th17 cells was significantly associated with faster time to doubling of creatinine and transplant loss; and the ratio of infiltrating Th17 cells over Foxp3$^+$ Treg cells was significantly associated with a decline in eGFR. These findings parallel and further support the published observations where the magnitude of Th17 cell infiltration over Treg cell infiltration correlated with kidney transplant dysfunction, the degree of interstitial inflammation and tubular atrophy, the refractoriness to treatment and the recurrence of ATCMR in the kidney transplant[21,22]. However, the associations observed in our study were not very strong. The observation that the numbers of infiltrating Th17 cells and the ratio of Th17 cells over Foxp3$^+$ Treg cells associated negatively with kidney transplant outcomes in the non-rejection patients was unexpected, but interesting. Alloimmune responses in transplant patients

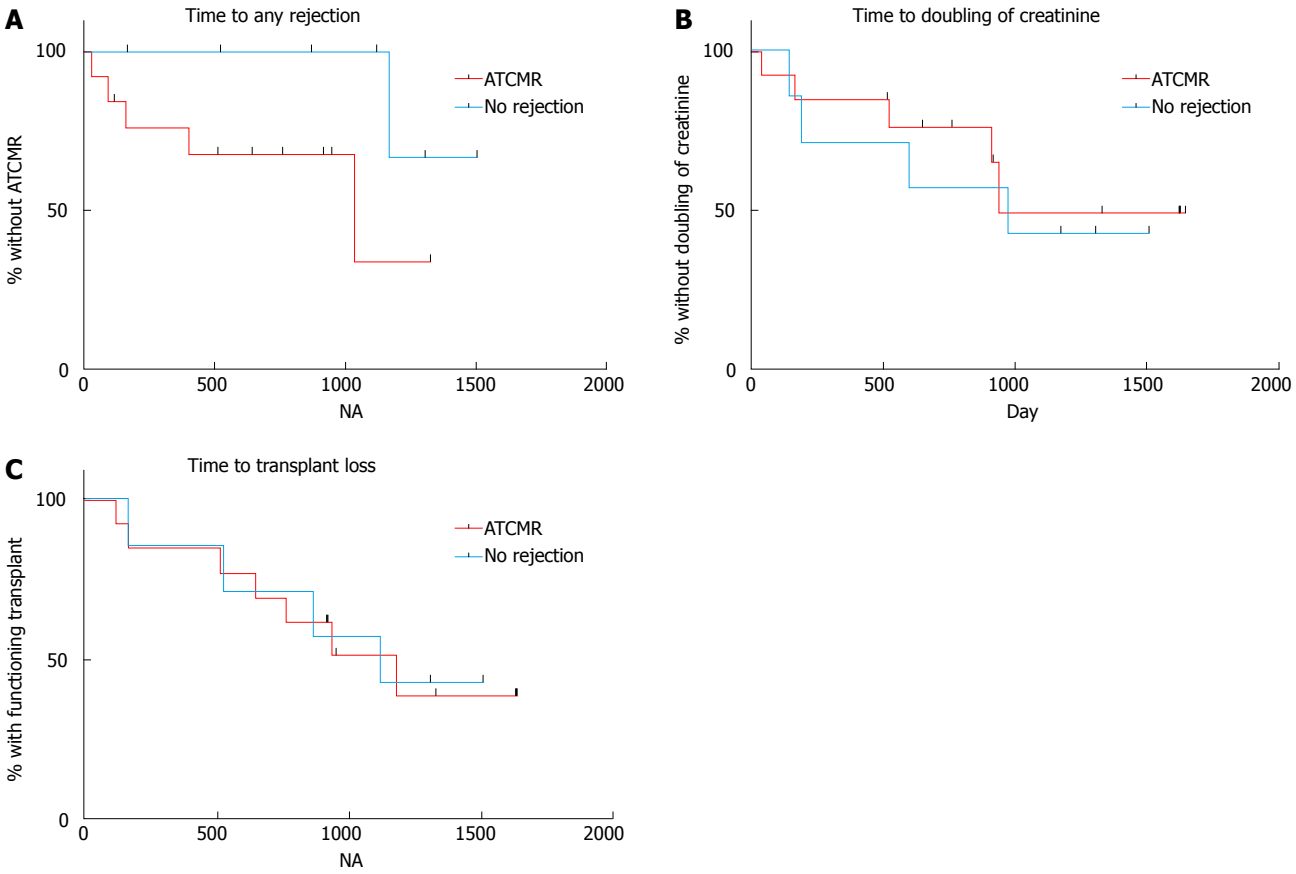

Figure 5 Time-to-event plots of (A) time to any rejection (borderline, acute T cell-mediated rejection in the kidney transplant or antibody-mediated rejection) post-biopsy, of (B) time to doubling of creatinine post-biopsy, and of (C) time to confirmed or suspected immune-mediated transplant loss in patients with acute T cell-mediated rejection in the kidney transplant (*n* = 14) and patients with no rejection (*n* = 7). Log-rank test *P* values for all the comparisons were statistically not significant. ATCMR: Acute T cell-mediated rejection.

Table 6 Effect of immune and clinical variables on kidney transplant outcomes

Outcomes	Risk factor	HR	95%CI	*P* value
Time to any rejection	Age	0.898	0.821, 0.983	0.0193
Time to doubling of creatinine	Infiltrating Th17 cells	1.031	1.002, 1.061	0.0359
Time to doubling of creatinine	Proteinuria	1.382	1.087, 1.757	0.0083
Time to transplant loss	Infiltrating Th17 cells	1.026	1.000, 1.052	0.0472
Time to transplant loss	Serum creatinine	1.009	1.003, 1.016	0.0036
Time to transplant loss	Delayed graft function	5.456	1.238, 24.036	0.0160

are detectable even in patients with apparent stable kidney function. Different sorts of immune cells are as a consequence "waiting for a chance" to flip over the silencing effects of maintenance immunosuppression and the deployed immunoregulatory mechanisms if "given the chance" (*i.e.*, reduction of immunosuppression, sensitizing events or the occurrence of concomitant infections or inflammatory disorders). Hence, it is possible that many transplants patients have certain degree of Th17 cell activation and infiltration. Thus, patients with higher degree of Th17 infiltration, irrespective of reaching the current thresholds for ATCMR-KTx or not, could be bound to worse outcomes due to the possibility that Th17 cells could be mediating smoldering inflammation or slow-motion chronic rejection or have the potential to mediate transformation into a rejection phenotype if the alloimmune milieu changes to a pro-inflammatory one. The use of more sophisticated technologies like the molecular microscope and a better classification of chronic T cell mediated rejection and i-IFTA (for inflamed areas of interstitial fibrosis and tubular atrophy) could help us in the future to assign a more accurate clinical significance to this interesting observation.

In contrast to published literature, in which a greater degree of infiltration by CTL in patients with ATCMR-KTx was associated with poorer allograft survival[18], and the magnitude of granzyme B expression was associated with the severity of the rejection process[10]; we found no association of CTL infiltration or the ratio of infiltrating CTL over Treg cells with kidney transplant outcomes.

However, we believe that statistical significance was not reached due to our small pilot sample size.

One of the merits of our study is the use of immunohistochemistry for our immunodetection as it is a highly available and inexpensive technology, easy to correlate to conventional light microscopy findings. Furthermore, in comparison to most available reports, our study provides a more comprehensive tissue staining, including the three markers that showed the best potential in the published literature: Granzyme B, IL-17 and Foxp3. Thus, our study hints that a more detailed immunohistochemical analysis of the cell infiltrate in kidney transplant biopsies can reflect more accurately the immune balance between the pro-rejection and anti-rejection forces and opens avenues for larger more powered and comprehensive confirmatory studies to address whether a detailed immunophenotyping of ATCMR-KTx can indeed improve the accuracy of the Banff classification; which is undergoing continuous improvement. It is important to comment that more sophisticated technologies like microarray technology have been used for the detection of CTL-associated transcripts and were reported to be more accurate than the detection of individual genes like perforin or granzyme B to cluster together patients with ATCMR-KTx[31]. However, this latter technology is not widely available and not as practical as immunohistochemistry; but indeed, microarray and high-throughput technologies such as the "omics" play a crucial role in biomarker discovery and identification of disease classifiers.

In addressing sample size, based upon our pilot study results, assuming a 1:2 sample size ratio of non-rejection:ATCMR-KTx patients, a common standard deviation (σ) and coefficient of variation (CV = $\sigma/\mu_{NoReject}$) 1.0 to 1.7, respective optimistic and pessimistic sample sizes to give 80% power to detect a two-fold ratio of CTL (CTL: Non-rejection/ATCMR-KTx $\geqslant$ 2) to Foxp3$^+$ Treg cells were calculated to be 18/36 (CV = 1.0) and 41/82 (CV = 1.7).

Participating patients were very heterogeneous in their clinical characteristics, which likely confounded our observations (Tables 1-3). For instance, we observed that the time to transplant loss from biopsy (not from transplant surgery) was similar in both patient groups. However, most kidney transplant biopsies in the non-rejection group were performed late post-transplantation, closer to their maximum transplant survival. In addition, the non-rejection group had higher proteinuria during the follow up period, which could be related also to their vintage in transplantation and likely higher degree of glomerulosclerosis, or perhaps proteinuria was an important factor in the decision to perform biopsy for those patients. Kidney transplant biopsies were indicated when transplant dysfunction ensued and recommended by treating nephrologists according to their own criteria and specific thresholds. The incorporation of selected immune parameters in a larger study including patients from the time of transplant surgery, subjected to more protocolised immunosuppressive regimens, or their incorporation in a clinical trial are anticipated to circumvent many of the biases in our study.

Finally, it would have been interesting to extend our protocol to assess the immune infiltrate inside the kidney transplant in protocol biopsies with subclinical ATCMR and without evidence of rejection. This could have helped us to address whether our observed immune changes mirror the events occurring in subclinical ATCMR-KTx, and to use negative protocol biopsies as better controls for a stable kidney transplant function. However, protocol biopsies are not performed in our institution.

The immune balance in ATCMR-KTx appears to be tilted numerically towards the pro-rejection forces, which seem to overwhelm counter-regulatory mechanisms. Similarly, the degree of infiltration of the kidney transplant by effector T cells could be associated with kidney transplant outcome prognosis. Although our findings are not conclusive, mainly due to our small sample size, they further elucidate the immunopathogenesis of ATCMR-KTx and open new avenues for a more detailed dissection of the complex immune mechanisms implicated in kidney transplant rejection. Upon further validation, ideally tested in randomised controlled trials, it is possible that these and other new signatures could be incorporated into the current diagnostic and therapeutic algorithms in order to deliver more personalised and precise management in kidney transplantation.

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COMMENTS

Background

In the clinical setting, acute T cell-mediated rejection in the kidney transplant (ATCMR-KTx) is only confirmed through a kidney transplant biopsy, which is scored according to the Banff classification. The Banff classification is largely based on the estimation of mononuclear cell infiltration instead of the identification and quantification of the actual T cell subsets recruited to mediate rejection.

Research frontiers

The identification of the actual T cell subsets involved in ATCMR-KTx likely

reflects more accurately the immune balance between effector and regulatory T cells, which has been implicated as an important factor determining the risk for ATCMR-KTx.

Innovations and breakthroughs

The detection of specific T cell subsets inside the kidney transplant suffering ATCMR adds new light to elucidate its immunopathogenesis, and opens new avenues for the development of novel biomarkers focusing on cytotoxic, Th17 cell-mediated and regulatory T cell responses.

Applications

A more detailed analysis of the inflammatory infiltrate of ATCMR-KTx, in particular of cytotoxic T lymphocytes and Th17 cells, is likely to enhance the diagnostic accuracy of the Banff classification.

Terminology

CD178: CD equivalent for Fas ligand, a membrane molecule able to trigger apoptosis upon ligation of CD95 in target allogeneic cells; Cytotoxic T lymphocytes: A subset of effector T cells able to cause direct cytotoxicity of transplanted parenchymal cells; Foxp3: Transcription factor crucial for the development and function of regulatory T cells; Granzyme B: Enzyme released by cytotoxic T lymphocytes able to trigger apoptosis in target transplanted cells; Regulatory T cells: A subset of T cells regarded as the master moderators of immune responses, thought to be able to regulate alloimmune responses and potentially to aid in the achievement of transplantation tolerance; Th17 cells: A subset of effector T cells implicated in the defence against exogenous microorganisms and implicated in the pathogenesis of several autoimmune disorders and effector alloresponses, whose characteristic cytokine product is IL-17.

Peer-review

This is a good article.

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ORIGINAL ARTICLE

Observational Study

Lymphocyte recovery is an independent predictor of relapse in allogeneic hematopoietic cell transplantation recipients for acute leukemia

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Author contributions: Damlaj M designed the study; Damlaj M, Ghazi S and Mashaqbeh W collected data; all authors analysed the data, provided patients, wrote and reviewed the manuscript, and approved final version of the manuscript.

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Abstract

AIM

To examine the optimal absolute lymphocyte count (ALC) cut-off utilizing receiver operator characteristics (ROC) in addition to graft characteristics associated with early ALC recovery.

METHODS

Patients who received T-cell replete peripheral hematopoietic cell transplantation (HCT) for acute leukemia were identified. ALC cut-off was established using ROC analysis and subsequently the cohort was stratified. Time to endpoint analysis and cox regression modelling was computed to analyze outcomes.

RESULTS

A total of 72 patients met the inclusion criteria and

were analyzed. Optimal ALC cut-off was established to be on day 14 (D14) with ALC > 0.3 × 10^9/L. At 2 years, cumulative incidence of relapse was 16.9% *vs* 46.9% (P = 0.025) for early and delayed lymphocyte recovery cohorts, respectively. Chronic graft *vs* host disease was more prevalent in the early lymphocyte recovery (ELR) group at 70% *vs* 27%, respectively (P = 0.0006). On multivariable analysis for relapse, ELR retained its prognostic significance with HR = 0.27 (0.05-0.94, P = 0.038).

CONCLUSION

ELR is an independent predictor for relapse in patients receiving allogeneic HCT for acute leukemia. ELR was influenced by graft characteristics particularly CD34 count.

Key words: Acute leukemia; Allogeneic transplant; Absolute lymphocyte count

Core tip: Disease relapse remains the most common cause of treatment failure after allogeneic hematopoietic stem cell transplantation for acute leukemia. Previous studies have identified that early lymphocyte recovery can be a surrogate of graft *vs* leukemia effect hence identifying high risk patients for relapse. However, published reports are heterogeneous with regards to timeline and magnitude of lymphocyte recovery. Using receiver operator characteristics with area under the curve, we identified that absolute lymphocyte count > 0.3 × 10^9/L at day 14 is associated with half the relapse risk which was statistically significant at the multivariable analysis. There was a trend towards improved progression free survival and overall survival for patients with early lymphocyte recovery. In conclusion, we observed that lymphocyte recovery is an independent predictor of relapse in allogeneic transplant recipients for acute leukemia. This would help identify high risk patients who may benefit from maintenance strategies post-transplant.

Damlaj M, Ghazi S, Mashaqbeh W, Gmati G, Salama H, Abuelgasim KA, Rather M, Hajeer A, Al-Zahrani M, Jazieh AR, Hejazi A, Al Askar A. Lymphocyte recovery is an independent predictor of relapse in allogeneic hematopoietic cell transplantation recipients for acute leukemia. *World J Transplant* 2017; 7(4): 235-242 Available from: URL: http://www.wjgnet.com/2220-3230/full/v7/i4/235.htm DOI: http://dx.doi.org/10.5500/wjt.v7.i4.235

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HCT) is widely used to cure a number of hematologic malignancies including acute myeloid leukemia (AML) and lymphoblastic leukemia/lymphoma (ALL)[1-4]. Relapse of the primary disease remains the most frequent cause of treatment failure in contemporary HCT recipients[5]. Several factors are associated with relapse such as status at HCT, associated cytogenetic abnormalities, conditioning regimen and occurrence of chronic graft *vs* host disease[6]. Prognosis after overt relapse post-HCT is very poor and a minority of patients are able to achieve durable remissions[7]. Hence, identification of patients at risk of relapse may permit preemptive interventions for relapse prevention[8].

Immune reconstitution post HCT, particularly lymphocyte recovery, can be a surrogate for graft *vs* leukemia (GVL) effect hence improved long term disease control. Several groups reported that early absolute lymphocyte count (ALC) recovery is associated with decreased relapse rates in hematologic malignancies. However, there is heterogeneity regarding the predictive optimal threshold and timing of lymphocyte recovery. For example Michelis *et al*[9] reported that ALC ⩾ 0.5 × 10^9/L on day 28 in AML patients is associated with reduction of the relapse risk at multivariable analysis with hazard ratio (HR) = 0.49 (0.26-0.92, P = 0.03) without a survival advantage. On the other hand, Kumar *et al*[10,11] showed that ALC ⩾ 0.15 × 10^9/L on day +30 resulted in a 3 fold reduction in relapse risk in AML patients but an ALC of > 0.17 × 10^9/L on day +21 was protective from relapse in ALL patients. Thoma et al. showed that ALC > 0.3 × 10^9/L on day +100 is associated with improved overall survival (OS)[12].

In light of the above discrepancies, we examined the impact of ALC recovery on post HCT outcomes; where optimal ALC threshold and timeline was analyzed using receiver operator characteristics (ROC) and area under the curve (AUC). We also analyzed infused allograft cellular content for factors predicting early ALC recovery.

MATERIALS AND METHODS

Patient selection

After due institutional review board (IRB) approval, patients ⩾ 14 years of age with AML or ALL who underwent HCT at our institution between 2010 - 2015 were identified.

The selection criteria included patients receiving myeloablative (MAC) or reduced intensity conditioning (RIC) from related or unrelated donors. Classification of the conditioning intensity was based on the criteria suggested by the Centre of International Blood and Marrow Transplant Research (CIBMTR)[13]. Selection of regimen intensity was at the discretion of the treating physician and generally patients with a hematopoeitic stem cell co-morbidity index (HCT-CI) < 3 were considered for MAC regimen[14]. Patients with ALL who were candidates for MAC, preferentially received a total body irradiation (TBI) based regimen. Exclusion criteria were for patients who received a bone marrow graft or cord blood stem cell source, second transplant and those who underwent *in vivo* or *in vitro* T-cell depletion. Data were collected retrospectively from the patient's electronic medical records. Cytogenetic data

at the time of diagnosis was collected and stratified as previously described for AML patients[15]. ALL patients with hypodiploid karyotype, translocations at (4;11), (11q23), (9;22) and (1;19) were deemed high risk, and remaining patients were classified as standard risk[16-20].

Preparative regimens and GVHD prophylaxis

Patients candidates for MAC intensity received one of two regimens based on the underlying diagnosis; patients with ALL received cyclosphosphamide 60 mg/kg intravenously (IV) for two days followed by 1200 cGy of TBI fractioned twice daily for three days. Patients with AML received fludarabine 30 mg/m^2 daily for five days in addition to busulfan 3.2 mg/kg IV daily for four days in addition to cyclophosphamide 60 mg/kg IV daily for 2 d. Mesna was given for bladder protection. For RIC regimens, patients received either fludarabine 30 mg/m^2 IV daily for 5 d with busulfan 3.2 mg/kg IV daily for two days or fludarabine 30 mg/m^2 IV daily for 5 d with melphalan 70 mg/m^2 IV for two days. Phenytoin loading and maintenance was given for seizure prophylaxis if busulfan was used until 24 h post last dose. Graft *vs* host disease (GVHD) prophylaxis consisted of methotrexate and cyclosporine. Methotrexate was given at 15 mg/m^2 on day +1 followed by 10 mg/m^2 on days +3, +6 and +11 with leucovorin rescue 24 h post each methotrexate dose. Day +11 was omitted if there is evidence of significant liver toxicity or grade $\geqslant$ 2 mucositis.

Definitions and transplant related outcomes

OS was calculated from the date of transplant until the date of death of any cause or last documented follow-up date. Progression free survival (PFS) was calculated from the time of transplant until death of any cause or relapse. Cumulative incidence of relapse (CIR) was calculated from the date of transplant until relapse or date of last follow up. Cumulative incidence of non relapse mortality (NRM) was calculated from the date of transplant until death of any cause without evidence of disease relapse. Acute and chronic GVHD was diagnosed according to standard criteria. Neutrophil engraftment was defined as an absolute neutrophil count (ANC) of 0.5 × 10^9/L or higher for 3 consecutive days. Platelet engraftment was defined as platelet count higher than 20 × 10^9/L for 7 consecutive days without transfusion support.

End points

The primary end point was to examine the impact of early ALC recovery (ELR) on CIR. Secondary endpoints were to examine effect of ELR on other post HCT outcomes (OS, PFS and NRM) and to examine infused allo-graft cellular content for factors predicting ELR. ALC was abstracted on days +7, +14, +21 and +28 from the Complete Blood Count (CBC) post HCT using either the automated or manual differential method[21].

Statistical analysis

Baseline patient, disease and treatment related variables were reported using descriptive statistics (counts, medians and percentages). Categorical and continuous variables were compared using Pearson's χ^2 and Wilcoxon/Kruskal-Wallis, respectively. Probability of OS was computed using the Kaplan-Meier method. Group comparisons were made using the log-rank test. Time to event was calculated from the date of transplant until the event of interest or point of last clinical encounter, in which case the event will be censored. Cumulative incidence was computed as competing events using Grey's model, considering death as a competing event for relapse and relapse as a competing event for NRM. Univariable and multivariable analyses were performed using Cox proportional hazard regression modelling and expressed as HR with 95%CI and *P* value. Any variable with a $P \leqslant 0.1$ was incorporated into the multivariable model in a stepwise selection process. Thresholds of ALC recovery post HCT as well as infused allograft characteristics, if present, were assessed using the ROC and AUC for the end point of relapse. Statistical analysis were performed using JMP Pro Version 11 (SAS Institute, Cary, NC, United States) software and EZR on R commander version 1.28[22].

RESULTS

Patient and transplant characteristics

A total of 72 patients met the inclusion criteria and their data were analysed. Baseline characteristics of the cohort are shown in Table 1. Majority of transplants were from related donors (88%), while the remaining minority (12%) were from unrelated donors. Transplants were from peripheral blood stem cells, while cord blood and bone marrow grafts were excluded due to different immune reconstitution kinetics. All patients were from the Middle East and North Africa Region. The median follow up was 17 mo (range: 2-64.8) at which point the CIR was 35.2% and OS was 67.3%.

Optimal ALC threshold

ROC curves with AUC were used to determine the best cut-off value for ALC on days +7, +14, +21 and +28 based on their utility as a marker for the binary outcome of relapse *vs* no relapse. ALC on day +14 > 0.3 × 10^9/L was identified as the optimal cut-off point. Patients were subsequently stratified as ELR if ALC on day +14 > 0.3 × 10^9/L and delayed lymphocyte recovery (DLR) if day +14 ALC was $\leqslant$ 0.3 × 10^9/L. Patient's disease and HCT related variables are stratified per lymphocyte recovery as shown in Table 1. Cohorts were similar with regards to age, gender, diagnosis, performance status, cytogenetic risk, status at HCT, stem cell source, donor gender, ABO matching and conditioning intensity. Regimens containing TBI were more common in the DLR group at 63% *vs* 33% ($P = 0.019$).

Infused allo-graft characteristics influencing ELR

We examined infused allo-graft cellular contents for factors predicting ELR in our patients. Optimal thresholds

Table 1 Baseline characteristics of patients stratified by lymphocyte recovery *n* (%)

Variable	ALC > 0.3 (*n* = 24)	ALC ⩽ 0.3 (*n* = 48)	*P* value
Patient age in years, median (range)	28 (16-57)	23 (14-63)	0.57
Recipient gender, male	13 (54)	28 (58)	0.74
Diagnosis			0.54
AML	13 (54)	22 (45)	
ALL	11 (46)	26 (54)	
ECOG	1 (0-2)	0 (0-3)	0.86
Cytogenetics (AML)			0.5
Favorable	3 (25)	2 (10)	
Intermediate	7 (58)	15 (71)	
High risk	2 (17)	4 (67)	
Cytogenetics (ALL)			0.78
Standard	5 (56)	11 (50)	
High risk	4 (44)	11 (50)	
Female donor/male recipient	4 (17)	11 (23)	0.53
Related donor	21 (88)	42 (88)	1
Status at HCT			0.33
CR1	13 (54)	31 (66)	
≥ CR2	11 (46)	16 (34)	
ABO Matching			0.89
Match	16 (67)	31 (64)	
Major/bidirectional	3 (12)	8 (17)	
Minor	5 (21)	9 (19)	
TBI containing regimen	8 (33)	30 (63)	0.019
Conditioning intensity			0.19
MAC	18 (75)	42 (88)	
RIC	6 (25)	6 (12)	

HCT: Hematopoietic stem cell transplant; AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia; ALC: Absolute lymphocyte count; ECOG: Eastern cooperative oncology group; TBI: Total body irradiation; CR: Complete remission; MAC: Myeloablative conditioning; RIC: Reduced intensity conditioning.

were again determined by ROC with AUC analysis. We observed that infusing grafts with the following characteristics was associated with higher incidence of ELR; CD 34 of < 6 × 10^6/kg (71% *vs* 42%, *P* = 0.018), CD3 > 24 × 10^7/kg (19% *vs* 2%, *P* = 0.017), infused ALC > 1.3 × 10^8/kg (96% *vs* 74%, *P* = 0.015), infused lymphocyte-monocyte ratio (LMR) > 4 (33% *vs* 11%, *P* = 0.022) and CD 34 < 6 × 10^6/kg with ALC > 1.3 × 10^8/kg (67% *vs* 27%, *P* = 0.0012). These results are show in Table 2.

Impact of ELR on post HCT outcomes

Stratified by lymphocyte recovery, after 2 years of follow up, the CIR was significantly higher for the DLR *vs* ELR groups at 46.9% *vs* 16.9%, respectively (*P* = 0.025). On the other hand, at 2 years, there was a non-significant difference of NRM between the two cohorts at 14.2% *vs* 23.3% for the DLR and ERL groups, respectively (*P* = 0.51). There was a trend towards improved 2 year PFS for the ELR at 61.9% *vs* 40.1% (*P* = 0.09), but no significant difference of OS was observed at 70.1% *vs* 53.9% for ELR *vs* DLR, respectively (*P* = 0.12) (Figure 1). Median time to ANC and platelet engraftment was similar for both groups at 17 (12-29) d and 24 (21-37) for ELR and 17 (12-25) and 24 (7-42), respectively (*P* = 0.76, 0.98). Incidence of aGVHD was similar but cGVHD was significantly higher in the ELR groups at 70% *vs* 27% (*P* = 0.0006). These results are shown in Table 3.

Table 2 Graft characteristics as predictors of lymphocyte recovery *n* (%)

Graft characteristic	ALC > 0.3 (*n* = 24)	ALC ⩽ 0.3 (*n* = 48)	*P* value
CD 34 × 10^6/kg < 6	17 (71)	20 (42)	0.018
TNC > 7 × 10^7/kg	5 (21)	10 (21)	1
CD 3 > 24 × 10^7/kg	4 (19)	1 (2)	0.017
CD 34 < 6 × 10^6/kg, CD 3 > 24 × 10^7/kg	3 (100)	0 (0)	0.0088
MNC > 2.7 × 10^8/kg	20 (83)	33 (69)	0.17
ALC > 1.3 × 10^8/kg	23 (96)	35 (74)	0.015
AMC > 1.75 × 10^8/kg	3 (13)	14 (30)	0.093
ALC > 1.3 × 10^8/kg, CD34 < 6 × 10^8/kg	16 (67)	13 (27)	0.0012
LMR > 4	8 (33)	5 (11)	0.022

ALC: Absolute lymphocyte count; TNC: Total nuclear count; MNC: Mononuclear count; AMC: Absolute monocyte count; LMR: Lymphocyte-monocyte ratio.

Six variables were found to influence relapse at univariable analysis; age at HCT HR = 0.97 (0.94-1.01, *P* = 0.1), single marital status HR = 2.59 (1.13-6.65, *P* = 0.023), female donor to male recipient HR = 2.15 (0.91-4.7, *P* = 0.079), CR1 remission HR = 0.52 (0.23-1.15, *P* = 0.1), cGVHD HR = 0.24 (0.079-0.59, *P* = 0.0013) and ELR 0.31 (0.09-0.8, *P* = 0.014). We also examined the impact of TBI on relapse given the higher incidence of TBI based conditioning in the DLR group, but did not see an apparent impact with HR = 1.003 (0.46-2.2, *P* = 0.99). Three factors remained prognostic at the multivariable analysis which were ELR HR = 0.27 (0.05-0.94, *P* = 0.038), CR1 remission HR = 0.36 (0.15-0.87, *P* = 0.024) and cGVHD 0.33 (0.1-0.92, *P* = 0.035). These results are shown in Table 4.

Causes of mortality in the ELR and DLR cohorts were related to relapse of primary disease in 3/8 (38%) and 18/24 (75%), infection 1/8 (12%) *vs* 0/24, organ failure 0/8 *vs* 1/24 (4.2%), aGVHD 1/8 (12) *vs* 2/24 (8.3%) and cGVHD 3/8 (38%) *vs* 3/24 (12%). These results are shown in Table 5.

DISCUSSION

The present analysis highlights again the value of ELR as a protective factor from disease relapse in acute leukemia. In particular, we report that ALC > 0.3 × 10^8/kg on day +14 post allogeneic HCT for acute leukemia is an independent factor predicting decreased CIR at multivariable analysis. We also observed a trend towards improved PFS and OS; however this did not meet statistical significance. NRM was not significant between both cohorts, however both the incidence of cGVHD and cGVHD related deaths were more frequent in the ELR group. Incidence of cGVHD related deaths

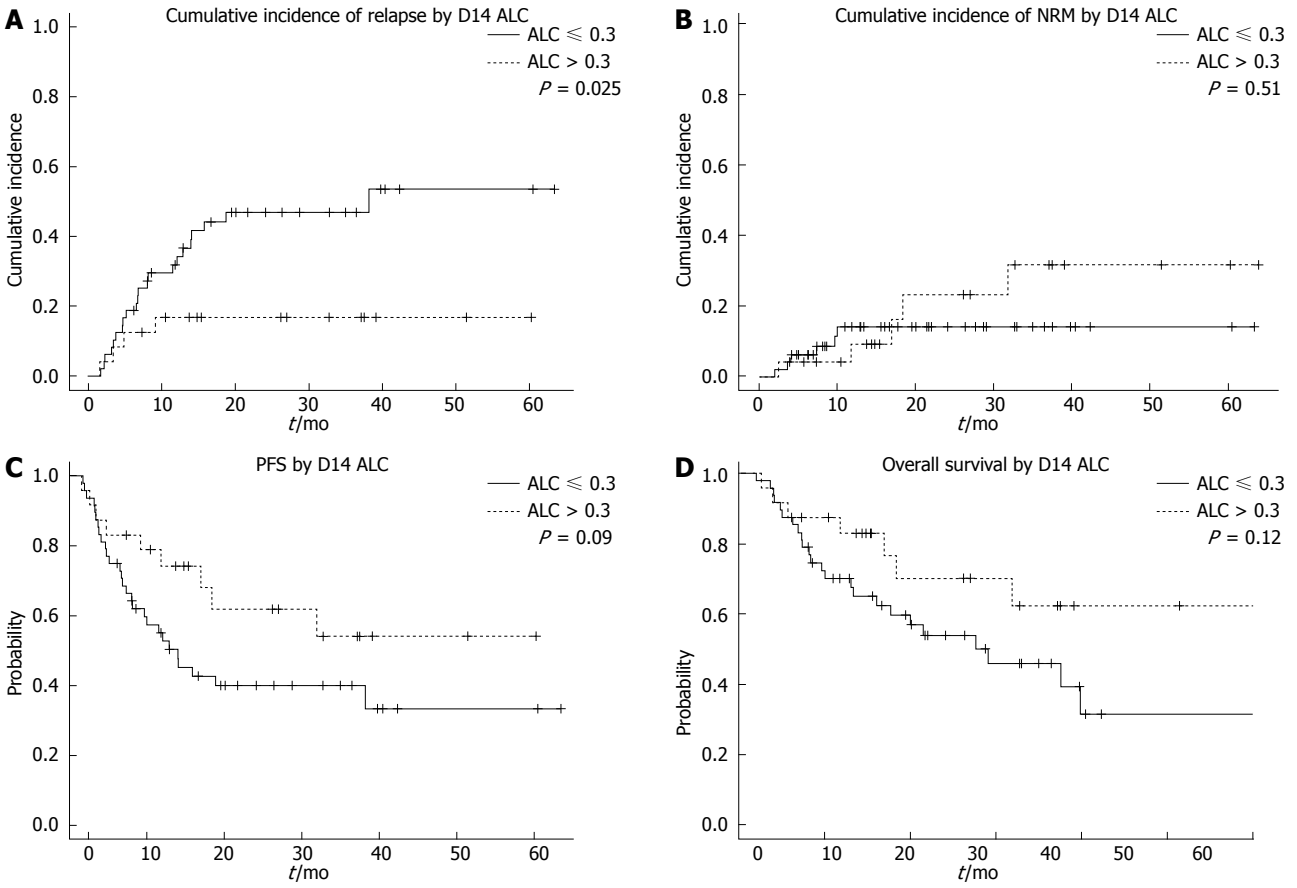

Figure 1 Post transplantation outcome of cumulative incidence of relapse (A), cumulative incidence of non-relapse mortality (B), progression free survival (C), and overall survival (D) stratified by lymphocyte recovery on day 14. ALC: Absolute lymphocyte count; PFS: Progression free survival.

Table 3 Transplant related outcomes

Variables	ALC > 0.3 (n = 24)	ALC ⩽ 0.3 (n = 48)	P value
CIR (2-yr)	16.90%	46.90%	0.025
NRM (2-yr)	23.20%	14.20%	0.51
PFS (2-yr)	61.90%	40.10%	0.09
OS (2-yr)	70.10%	53.90%	0.12
Plt engraftment (median, d)	24 (21-37)	24 (7-42)	0.98
ANC engraftment (median, d)	17 (12-29)	17 (12-25)	0.76
aGVHD	5 (22)	15 (31)	0.4
cGVHD	16 (70)	13 (27)	0.0006

ALC: Absolute lymphocyte count; CIR: Cumulative incidence of relapse; NRM: Non-relapse mortality; PFS: Progression free survival; OS: Overall survival; Plt: Platelet; ANC: Absolute neutrophil count; aGVHD: Acute or chronic graft *vs* host disease; cGVHD: Chronic graft *vs* host disease.

was 37.5% (3/8) in the ELR group compared to 12.5% (3/24) in the DLR group. This perhaps explains the lack of statistical significance seen for PFS and OS.

Give that graft source and manipulation can affect cellular reconstitution post-transplant, we excluded patients who received bone marrow or cord blood grafts in addition to those receiving T-cell depleted manipulation of the graft[23,24]. TBI was administered more frequently in the DLR group, but we did not observe an impact on relapse using TBI at the univariable analysis level with HR: 1 (0.46-2.2, P = 0.99).

At multivariable analysis, three factors had an impact on relapse: CR1, cGVHD and ELR. cGVHD is well described to decrease incidence of relapse due to a parallel GVL effect[25]. The current analysis supports the hypothesis that ELR is a surrogate for GVL as cGVHD incidence was significantly higher in the ELR group. Incidence of cGVHD related deaths were also more frequent in the ELR group, which likely accounts for the observed NRM, PFS and OS rates.

Although lymphocyte subsets were not identified in this analysis, the most plausible subset implicated in our analysis would likely be the natural killer (NK) cells as they represent the bulk of recovered lymphocytes by two weeks post HCT[26]. Previously, NK cells were found to be an independent factor predicting post HCT outcomes in T-cell depleted grafts[27]. However, this finding was not reproduced when T-cell replete grafts were used[28]. That said, this observed protective effect from ELR is likely a complex interplay between various lymphocyte subsets, such as NK cells, cytotoxic T-lymphocytes (CD8+) and regulatory T-cells (CD4+ and CD25+)[29,30]. Furthermore, the infused graft cellular content likely impacts post HCT reconstitution, and this has been well demonstrated in the autologous HCT setting and to a lesser extent allogeneic HCT[12,31-33].

Infused allo-graft cellular content predicts post HCT reconstitution. We observed that higher T-cell

Table 4 Univariable and multivariable risk factors influencing incidence of relapse

IR	Univariable HR (95%CI, *P* value)	Multivariable HR (95%CI, *P* value)
Age at HCT	0.97 (0.94-1.01, $P = 0.1$)	0.13 (0.0096-1.38, $P = 0.093$)
Single Marital status	2.59 (1.13-6.65, $P = 0.023$)	0.82 (0.21-3.27, $P = 0.77$)
AML *vs* ALL	0.82 (0.36-1.8, $P = 0.62$)	
Female D *vs* Male R	2.15 (0.91-4.7, $P = 0.079$)	2.24 (0.88-5.31, $P = 0.086$)
Match *vs* Mismatch	1.9 (0.3-6.7, $P = 0.42$)	
MRD *vs* Other	1.6 (0.47-10, $P = 0.49$)	
D14 ALC > 0.3	0.31 (0.09-0.8, $P = 0.014$)	0.27 (0.05-0.94, $P = 0.038$)
MAC *vs* RIC	1.38 (0.46-3.4, $P = 0.53$)	
CR1 *vs* other	0.52 (0.23-1.15, $P = 0.1$)	0.36 (0.15-0.87, $P = 0.024$)
aGVHD	0.54 (0.16-1.43, $P = 0.23$)	
cGVHD	0.24 (0.079-0.59, $P = 0.0013$)	0.33 (0.1-0.92, $P = 0.035$)

ALC: Absolute lymphocyte count; HR: Hazard ratio; CR1: First complete remission; R: Recipient; D: Donor; AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia; cGVHD: Chronic graft *vs* host disease; MAC: Myeloablative conditioning; RIC: Reduced intensity conditioning; MRD: Matched related donor.

Table 5 Causes of mortality stratified by absolute lymphocyte count recovery

Variables	ALC > 0.3 ($n = 8$)	ALC ⩽ 0.3 ($n = 24$)
Primary disease	3	18
Infection	1	N/A
Organ failure	N/A	1
aGVHD	1	2
cGVHD	3	3

ALC: Absolute lymphocyte count; aGVHD: Acute or chronic graft *vs* host disease; cGVHD: Chronic graft *vs* host disease; N/A: Not available.

and absolute lymphocyte content was significantly associated with ELR. Higher CD34 content is typically associated with faster engraftment and decreased rejection[34,35]. The National Marrow Donor Program (NMDP) reported on a cohort of over 900 unrelated HCTs using peripheral blood stem cells indicating that higher CD34 doses resulted in rapid engraftment, decreased transplant related mortality (TRM) and improved OS using various conditioning regimens[36]. However, the median stem cell dose administered was 6×10^6/kg and 5×10^6/kg in myeloablative (MAC) and reduced intensity RIC transplants, respectively. We found that infusing $< 6 \times 10^6$/kg stem cells was significantly associated with ELR. This is consistent with other reports indicating that administering higher doses of stem cells leads to detrimental outcomes both in MAC and RIC regimens[37-40]. Collectively, it appears that the optimal stem cell dose is 6-8 $\times 10^6$/kg, thus striking a balance between (GVL) and GVHD[41].

This analysis has inherent limitations, primarily due to the retrospective nature and sample size. We excluded patients who had T-cell manipulation or grafts other than peripheral blood stem cells as these factors can impact immune reconstitution. However, a number of important observations were made. First, similar to prior reports, we observed that ELR is protective of relapse but the timing post HCT and lymphocyte thresholds were determined using ROC-AUC and not empirically. Second, a higher incidence of cGVHD and cGVHD related deaths was seen with ELR, which confirms the likely mechanism of lower CIR seen in this cohort. Interestingly, marital status was significantly associated with decreased CIR although it did not retain significance at the multivariable analysis. Lastly, we reported that infusing less stem cells correlates better with ELR thus challenging the notion of "more is better".

In summary, the presented study demonstrates an independent protective effect of ALC at 14 d post allogeneic HCT. Given that patients with acute leukemia relapsing after allogeneic HCT have a dismal prognosis. Early identification of these cases may facilitate pre-emptive decisions such as early cessation of immune-suppression or use of lymphocyte infusion in order to better harness the GVL effect, or other maintenance strategies such as hypomethylating agents. These important observations warrant further study.

COMMENTS

Background

Disease relapse remains the most common cause of treatment failure after allogeneic hematopoietic stem cell transplantation for acute leukemia. Several factors are associated with relapse such as status at hematopoietic cell transplantation (HCT), associated cytogenetic abnormalities, conditioning regimen and occurrence of chronic graft *vs* host disease. Prognosis after overt relapse post-HCT is very poor and a minority of patients are able to achieve durable remissions. Hence, identification of patients at risk of relapse may permit preemptive interventions for relapse prevention. Immune reconstitution post HCT, particularly lymphocyte recovery, can be a surrogate for GVL effect hence improved long term disease control.

Research frontiers

Several groups reported that early absolute lymphocyte count (ALC) recovery is associated with decreased relapse rates in hematologic malignancies. However, there is heterogeneity regarding the predictive optimal threshold and timing of lymphocyte recovery.

Innovations and breakthroughs

The authors examined the impact of ALC recovery on post HCT outcomes. Using receiver operator characteristics with area under the curve, the authors identified that absolute lymphocyte count > 0.3×10^9/L at day 14 is associated with half the relapse risk which was statistically significant at the multivariable analysis. The authors also observed that infused graft content influences ALC

recovery.

Applications

Given that patients with acute leukemia relapsing after allogeneic HCT have a dismal prognosis. Early identification of these cases may facilitate pre-emptive decisions such as early cessation of immune-suppression or use of lymphocyte infusion in order to better harness the GVL effect, or other maintenance strategies such as hypomethylating agents.

Terminology

ALC recovery post allogeneic HCT is an easy to measure marker and can be used as a surrogate to identify high risk patients for relapse. Using receiver operator characteristics with area under the curve can help identify the optimal ALC threshold to exhibit this protective effect.

Peer-review

This is an interesting study, demonstrating lymphocyte recovery as independent predictor for relapse in allogenic hematopoietic stem cell transplantation for acute leukemia.

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CASE REPORT

De novo intraocular amyloid deposition after hepatic transplantation in familial amyloidotic polyneuropathy

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Author contributions: Gama IF and Almeida LD designed the report; Almeida LD evaluated and followed-up the patient; Gama IF performed the complimentary exams and collected all clinical data; Gama IF and Almeida LD wrote the paper.

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Abstract

The familiar amyloid polyneuropathy (FAP) is a rare autosomal-dominant systemic amyloidosis. Amyloid deposition occurs more frequently and extensively in the vitq. The increase in intraocular pressure (IOP) is a result of deposition of transthyretin (TTR) in trabecular meshwork. Rarely, the amyloid deposition in anterior segment can be more exuberant than in posterior segment. A 42 years old man, with FAP (Val30Met mutation), liver transplantation in 1997. He was asymptomatic, without any significant ocular abnormality until 2011. In 2011 he had an episode of pain in right eye (RE). Scalloped pupils, pupillary amyloid deposits and subtle vitreous opacities were detected. The IOP was 40 mmHg in RE and 28 mmHg in left eye (LE) with open angle. Optical coherence tomography detected a temporal superior retinal nerve fiber layer defect in LE and perimetry was normal. Topical timolol was initiated, and brimonidine was subsequently added to improve IOP control, which was achieved with topical medication until last evaluation. No progression occurred since 2011. Actually, with longer life expectancies, there is an increased risk of ocular involvement in FAP, even after liver transplantation. Although rare, a more exuberant amyloid deposition in anterior segment *vs* posterior segment can occur, and supports an important role of amyloid production in ciliary pigment epithelium in these patients. Medical control of IOP and a stable course are unusual in this secondary glaucoma. Ophthalmologists have an important task in the follow-up of patients and early diagnosis of risk factors for secondary glaucoma, such as scalloped pupils with amyloid deposits.

Key words: Familial amyloid polyneuropathy; Glaucoma; Scalloped pupils; Pupillary amyloid deposits; Liver transplantation

Core tip: Ocular manifestations of familial amyloidotic

polyneuropathy (FAP) can appear after liver transplantation due to *de novo* ocular production of amyloid. Rarely, amyloid deposition in vitreous is relatively less exuberant than in anterior segment. Our case illustrates this asymmetry of amyloid deposition and emphasizes the association between scalloped pupils and glaucoma, a major ocular complication of FAP. Our case had a stable course, with excellent visual function and the intraocular pressure was controlled by medical therapy, which are unusual in this type of glaucoma. This case-report also highlights the importance of the long-term ophthalmological follow-up in FAP patients.

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INTRODUCTION

Transthyretin (TTR)-related familial amyloid polyneuropathy (FAP) is a group of autosomal-dominant diseases of variable penetrance caused by the deposition of polymerized mutated TTR in the peripheral nerves, gastrointestinal tract, heart, ocular tissues, and other organs. These protein aggregates have affinity for Congo red stain and apple-green birefringence when viewed under polarized light[1-3]. They are caused by mutations of TTR gene (18q11.2-12.1)[2]. Peripheral neuropathy is progressive and frequently the first manifestation of the disease[1-3]. Type 1 FAP, the Portuguese type (FAP1) was described for the first time in 1952 by Corino de Andrade[4]. FAP1 is the most frequent type of FAP and caused by a mutational substitution of the valine for methionine in position 30 of TTR gene (Val30Met)[2].

There are many ophthalmological manifestations of FAP caused by deposition of amyloid in various intraocular tissues: Vitreous, iris, pupillary border, anterior capsule and trabecular meshwork. The pupillary margin may have a scalloped/indented configuration (scalloped pupils) and pupils may be slow or nonreactive to both light and near stimulation, caused by disturbance of autonomic innervation[1-3,5-8]. Fleck deposits resembling pseudoexfoliation (PEX) may be found on the anterior lens capsule and pupillary margin[1-3,7]. *Pseudopodia lentis* is a hallmark of vitreous amyloidosis, where multiple small dots or footplates are formed on the posterior lens surface[2]. Trabecular meshwork deposition of amyloid causes obstruction of aqueous humor outflow and subsequent elevation of intra-ocular pressure (IOP)[9]. Secondary glaucoma can develop rapidly with high IOP, which if left untreated it can lead to severe damage[3,9]. Other manifestations include dry eye by decreased tear production, conjunctival microaneurysms and reduced corneal sensitivity with subsequent neurotrophic corneal ulcers[2].

TTR is a normal constituent of blood plasma, acts as a thyroxine transport protein and is important in vitamin A transport[2,3]. TTR is synthesized mainly in liver (90%), but there is also intra-ocular production[1,3,6,7,10-12]. Retinal pigment epithelium (RPE) is a source of TTR synthesis in rat eyes[10]. Recently, it was demonstrated that TTR production also occurs in the ciliary pigment epithelium (CPE)[12].

Liver transplantation (LT) improved the quality and survival of FAP patients, but does not prevent ocular manifestations of FAP, because of persistent intra-ocular production of amyloidotic TTR (ATTR). A case of vitreous amyloidosis appearing 2 years after LT was described and mutant protein ATTR was detected in aqueous humor of a Japanese patient after LT[13,14].

Secondary glaucoma is a major complication of FAP, which can be the first ocular manifestation and cause irreversible visual loss. Thus, early diagnosis is fundamental to avoid rapid progression of glaucoma[9].

The authors want to emphasize the importance of the recognition of ophthalmological signs that are associated with increased risk of ocular hypertension and glaucoma in FAP1 patients after LT as well as to report an unusual asymmetric pattern of intraocular amyloid deposition, with a case report and bibliographic revision.

CASE REPORT

A 42-year-old man had a diagnosis of FAP since 1995, with a positive genetic test for ATTR Val30Met mutation, and was subjected to LT in 1997. The peripheral neuropathy improved after LT. His brother and mother had type 1 FAP. The patient did not have any other previous ophthalmological diagnosis besides myopia. No FAP-related ophthalmological abnormalities were detected on routine ophthalmology evaluations for 14 years after LT.

In November 2011, he had an episode of ocular pain in right eye (RE) and attended the emergency room. Best-corrected visual acuity was 20/20 in RE and left eye (LE). Pupils were isochoric with slow pupillary responses to light and near stimulation. Ocular movements were normal. Biomicroscopy showed bilateral whitish fleck flocculent deposits of amyloid in the pupillary borders, scalloped pupils and few deposits in anterior vitreous (Figure 1). The detection of abnormalities led to the measurement of intraocular pressure (IOP) by Goldmann applanation tonometry (GAT), being 40 mmHg in RE and 28 mmHg in LE. Gonioscopy showed open angles - Shaffer grade of 4. Fundoscopy and retinography showed few vitreous opacities and clearly visible normal posterior poles, with normal appearing optic discs (Figures 2 and 3). Central corneal thickness was 559 μm in RE and 550 μm in LE. Ophthalmic ultrasound (US) showed few vitreous opacities bilaterally (Figure 4). Optical coherence tomography (OCT) only showed a superior-temporal

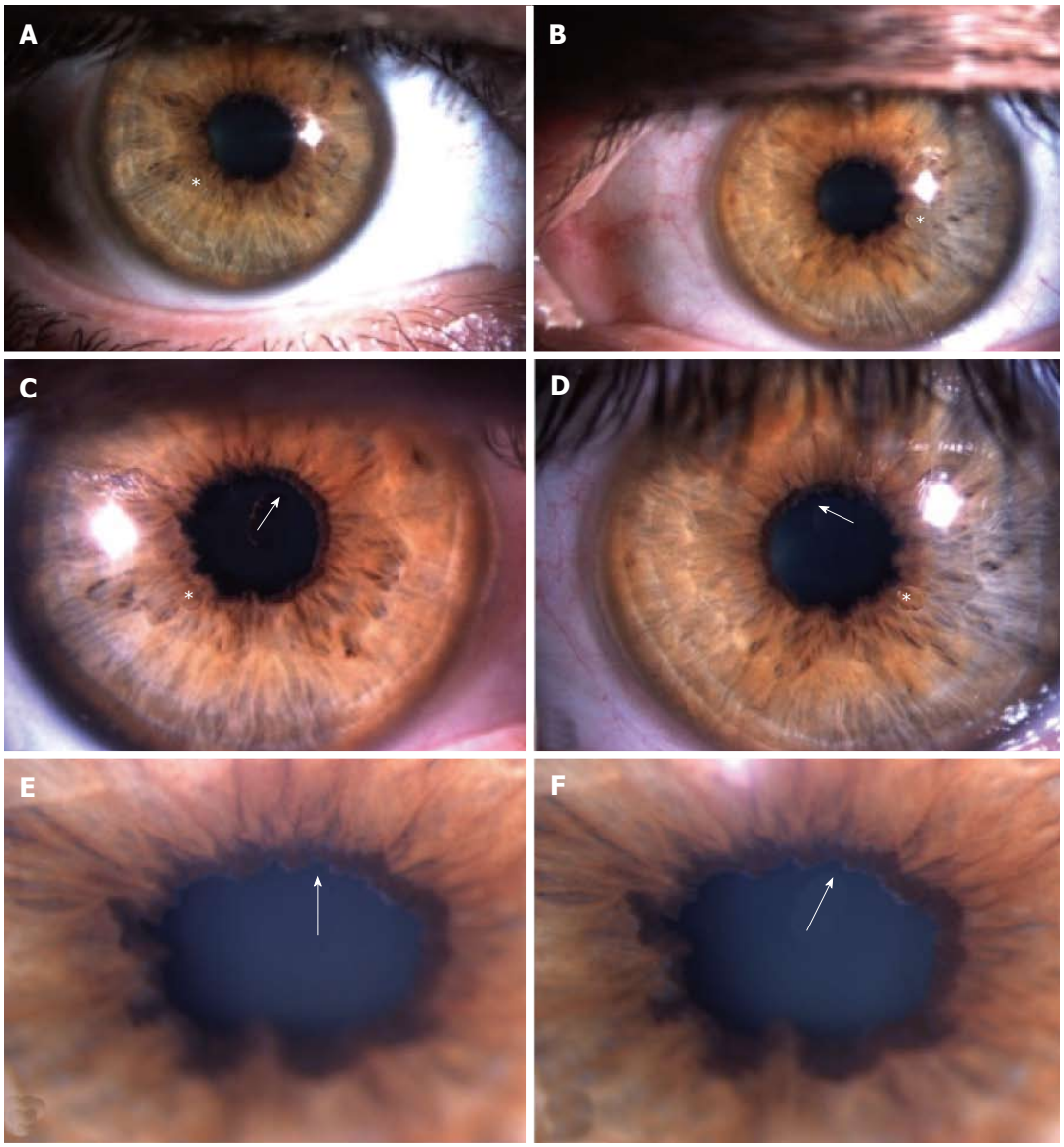

Figure 1 Slit-lamp photos showing scalloped pupils (astherisks) and amyloid deposition in the pupillary border (arrows) in both eyes. A and B: Slit-lamp photos of anterior segment of right (A) and left eyes (B) at low magnification; C and D: Slit-lamp photos at higher magnification to show pupillary margins of right (C) and left (D) eyes with more detail, in order to highlight the irregular pupillary margins, the scalloped pupils (astherisks) with amyloid deposits (arrows); E and F: Slit-lamp photos of the right eye (E and F) at the highest magnification to enhance visualization of the pupillary amyloid deposits (arrows), which resemble those seen in pseudoexfoliation syndrome.

peripapillary retinal fiber layer retinal nerve fiber layer (RNFL) defect in LE (Figure 5). Automated perimetry was unremarkable in both eyes (Figure 6). Topical monotherapy with timolol 0.5% was initiated at that time, and the IOP lowered to 26 mmHg in RE and to 21 mmHg in LE. To optimize IOP control, brimonidine was associated with timolol further lowering the IOP to 14 mmHg in both eyes. The patient was followed up closely in the glaucoma clinic until present, with controlled IOP. Last CSP and OCT exams excluded glaucoma progression.

DISCUSSION

Although ATTR levels after LT decline to < 1% of pre-transplant levels, FAP patients are still at risk of

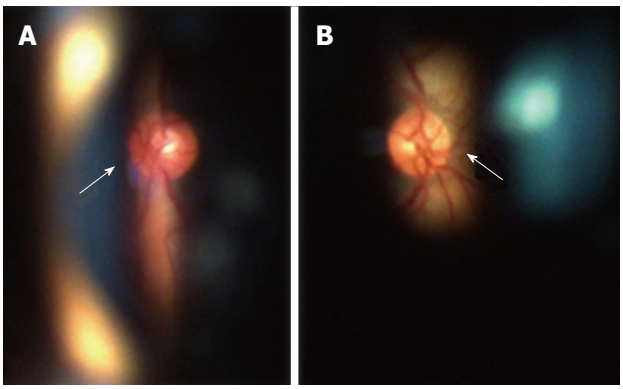

Figure 2 Fundoscopy of right (A) and left (B) eyes showed normal-appearing optic discs (arrows) and absence of abnormalities in posterior pole and peripheral retina. Ocular fundus was perfectly visible due to mild amyloid deposition in the vitreous.

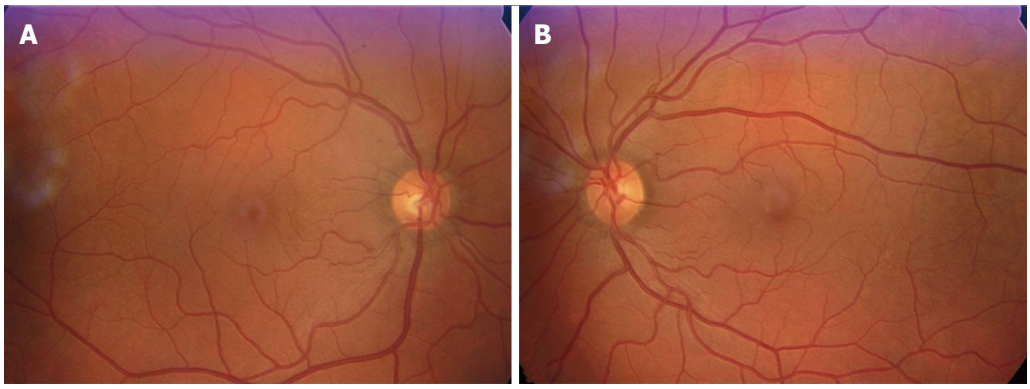

Figure 3 Retinography of right eye (A) and left eye (B) showed normal posterior poles, which were clearly visible due to the mild amyloid deposition in the vitreous, with only few opacities, which did not compromise visual acuity.

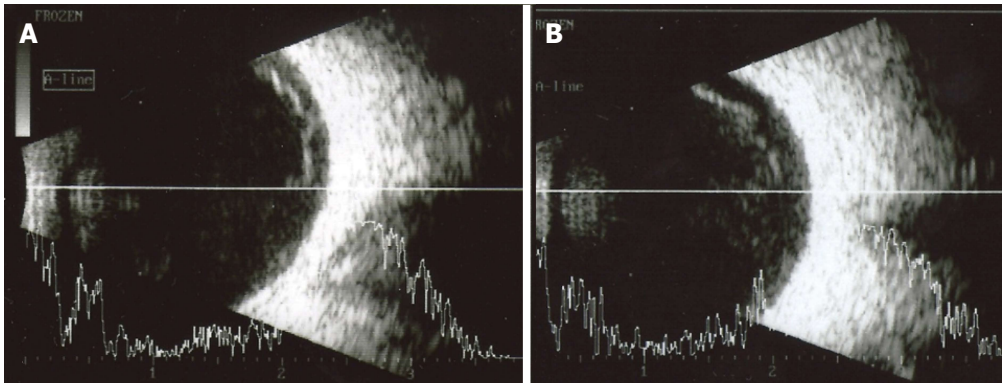

Figure 4 Ophthalmic ultrasound of right (A) and left (B) eyes showing some vitreous opacities corresponding to amyloid deposits in the vitreous. This amyloid deposition in the vitreous is relatively mild compared to the degree of amyloid deposition in anterior segment.

ophthalmological complications of the disease because of continued intra-ocular production in RPE and CPE and continued amyloid deposition in various ocular tissues, such as vitreous, pupil, anterior lens capsule and trabecular meshwork[1,3,6,7,10,12-18].

LT improved survival and consequently there is increased risk of ocular complications of FAP in transplantation era, because ocular manifestations are dependent of the duration of systemic disease. Glaucoma is a major ocular complication of FAP and a major cause of visual loss in these patients[1,3,19,20].

The study of Kimura *et al*[1] reported glaucoma in 24% of all FAP patients and in 17% of patients with Val30Met mutation, but the prevalence of glaucoma differs in various studies, from 5.4% to 27%[1,20,21]. Glaucoma is secondary to amyloid deposition in trabecular meshwork and if not recognized or treated adequately can have a rapid progression and devastating visual consequences in these patients, who have already a great morbidity from the systemic disease[1,3,19,20]. The pathophysiology of glaucoma in FAP1 after LT is related to the deposition of amyloid fibrillar aggregates in intertrabecular spaces of corneoscleral and uveoscleral meshworks and degeneration of endothelium cells of trabecular meshwork[6]. The trabecular outflow resistance increases, which raises IOP. Perivascular deposition of amyloid in conjunctival and scleral tissues can increase episcleral pressure and, consequently, the outflow resistance, but this mechanism is mainly dependent on systemic production of ATTR, playing an important role only before LT[6].

Most of the ophthalmological studies of FAP are focused in vitreous opacities (VO) and there are only few studies about secondary glaucoma. In the study of Kimura *et al*[1], VO were found in 35% of patients and amyloid deposition in pupil and anterior lens capsule in 31% of patients. Scalloped pupils are caused by autonomic abnormalities, which are associated with a higher degree of amyloid deposition in anterior segment and can also predict glaucoma[3]. They occur in 8% of FAP patients and glaucoma in 20% of patients. Glaucoma was diagnosed in all cases (100%) with scalloped pupils and in 57% of cases with amyloid deposition in anterior segment (pupil and anterior lens capsule). Only 49% of cases with VO had glaucoma[1]. Vitreous opacities are a classic ocular manifestation of FAP, but accordingly to the studies of Kimura *et al*[1] and Sandgren *et al*[3], the association between VO and glaucoma is weaker than between glaucoma and pupillary abnormalities (scalloped pupils, ATTR deposition in pupillary margin)[1,3]. This finding is supported by our clinical case.

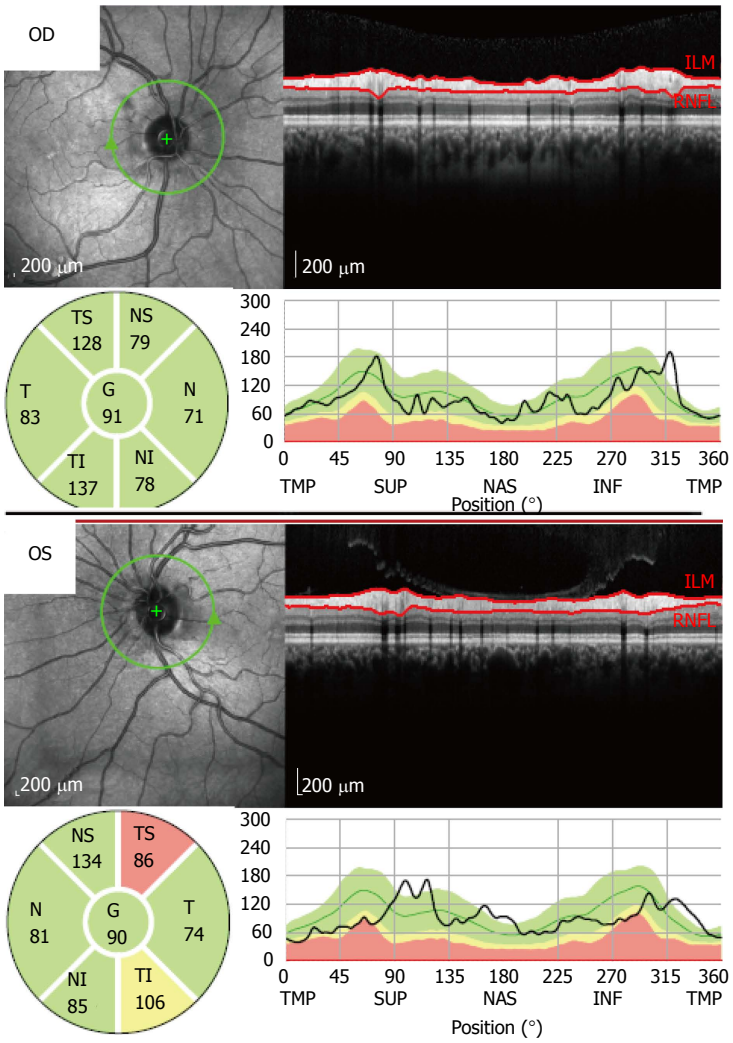

Figure 5 Optical coherence tomography showed a localized defect in the temporal-superior area of the peripapillary retinal nerve fiber layer of the left eye (OS). In the right eye (OD), the retinal nerve fiber layer thickness was normal in all peripapillary locations.

Sandgren *et al*[3,7] suggested that amyloid deposits in pupil and anterior lens capsule are more precocious than in the vitreous, which can explain the existence of rare cases, such as our case, which have much more ATTR deposition in anterior segment than in vitreous. These rare cases, such as our clinical case, corroborate the hypothesis raised by the study from Kawaji *et al*[12] postulating that the ATTR accumulated in anterior segment may have origin in CPE. This hypothesis can explain this asymmetry between ATTR deposition in anterior and posterior segments, as occurred our clinical case[12].

Amyloid is transported in the aqueous. Thus, pupillary amyloid deposits are an indirect sign of exuberant amyloid deposition in anterior segment, including the trabecular meshwork. This results in an increased resistance to aqueous humor outflow[19]. Kimura *et al*[1] have found that pupillary amyloid deposits have preceded the diagnosis of glaucoma by an average period of 2.55 ± 1.43 years (range 0.2-4.0 years). In the presented clinical case, the recognition of the pupillary abnormalities raised the clinical suspicion of glaucoma that was confirmed by appropriate investigation. Preperimetric glaucoma was confirmed by the finding of a localized defect of nerve fiber layer without perimetric functional repercussion.

Most cases of glaucoma secondary to FAP are usually refractory to medical treatment and have a fast progression and bad prognosis. This type of glaucoma usually requires surgical treatment[8]. Tube shunts, specially the Ahmed valve have been extensively used for surgical treatment of FAP1-related glaucoma in Portugal[8]. Recently minimal invasive options for glaucoma treatment are available for primary open-angle glaucoma and some types of secondary glaucoma, having the advantage of being less traumatic to the eye. However, prospective studies of efficacy in FAP-related glaucoma are lacking. Our clinical case had an unusual clinical course, with a good IOP control with medical treatment and stable visual fields and RNFL thicknesses.

Pars plana vitrectomy can be performed if vitreous opacities impair visual acuity, but this was not the case of our patient. Also, glaucoma can occur or be aggravated after pars plana vitrectomy in FAP patients, which is an important aspect to consider when managing ocular manifestations of FAP patients also affected by secondary glaucoma.

In an era that FAP patients have a greater life expectance with liver transplant, there is an increased probability of serious ocular disease caused by FAP, such as glaucoma that requires a regular ophthalmologic follow-up.

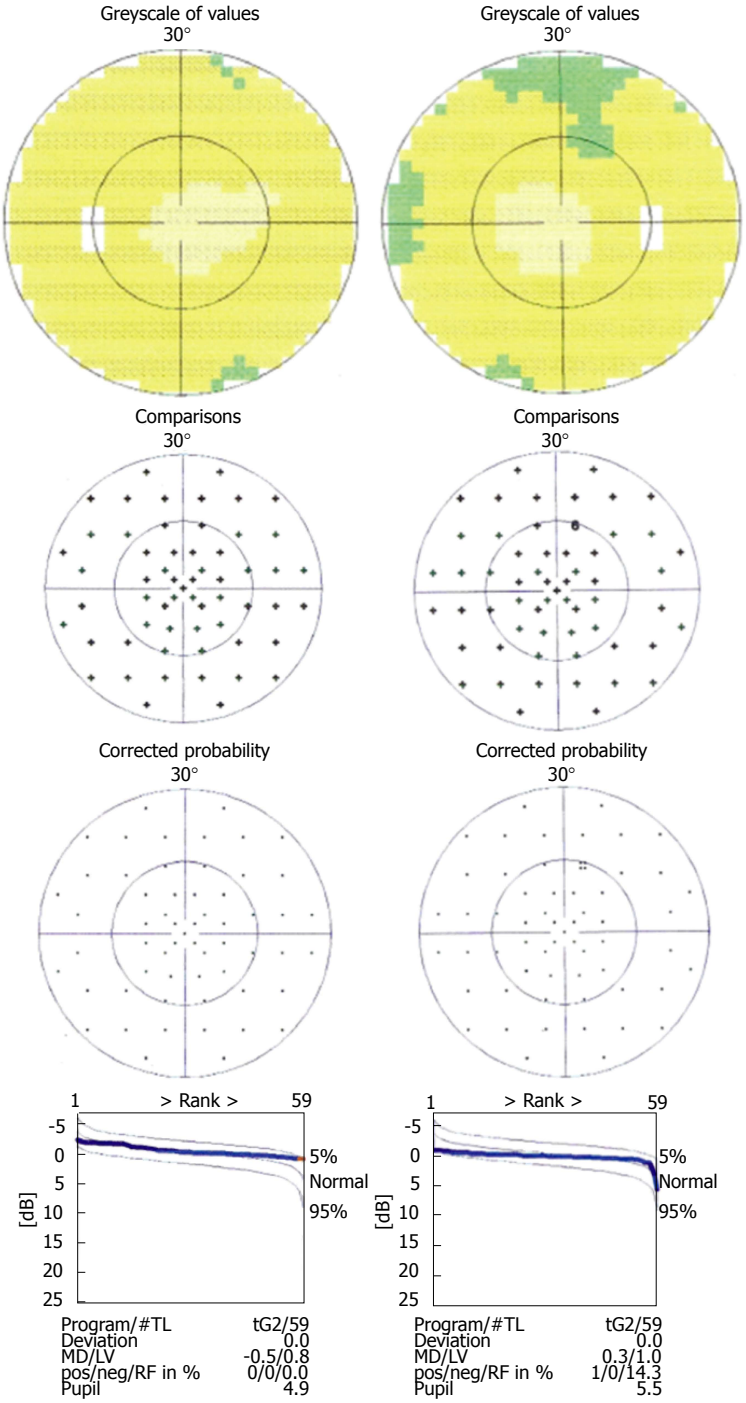

Figure 6 Computerized static perimetry of right eye (at the right) and left eye (at the left) (tendency-oriented perimetry, TOP - 30º program, Octopus 101 perimeter, Haag-Streit Diagnostics, Switzerland) in November 2011, showed the absence of clinically significant abnormalities in the visual fields - preperimetic glaucoma.

COMMENTS

Case characteristics

A 41-year-old man with type 1 familiar amyloid polyneuropathy (FAP) subjected to liver transplantation in 1997, presented with ocular pain.

Clinical diagnosis

Ophthalmological examination showed ocular hypertension, scalloped pupils associated to exuberant amyloid pupillary deposits, which contrasted with the mild vitreous opacities on ultrasound.

Differential diagnosis

FAP-related secondary open-angle glaucoma, FAP-related secondary ocular hypertension, pseudoexfoliation glaucoma, ocular hypertension associated to pseudoexfoliation syndrome.

Imaging diagnosis

Ocular ultrasound showed mild vitreous opacities due to amyloid deposition. Retinography showed normal posterior poles. Optical coherence tomography only showed a peripapillary temporal-superior retinal nerve fiber layer defect in OS. Perimetry did not show significant visual field abnormalities.

Treatment

Treatment with topical timolol and brimonidine achieved intraocular pressure (IOP) control. This treatment was continued, permitting disease stabilization with IOP control. This is a rare clinical course of this disease.

Related reports

De novo intraocular amyloid synthesis and deposition occurs after liver transplantation, having the potential to cause serious ocular complications. Most reported cases of FAP-related secondary glaucoma with scalloped

pupils have exuberant vitreous amyloid deposition. The asymmetry between exuberant amyloid deposition in anterior segment *vs* mild vitreous deposition that was reported in this clinical case is rare, and suggests a role of ciliary pigment epithelium in intraocular amyloid synthesis. This clinical case had a rare clinical course.

Term explanation

FAP-related glaucoma after liver transplantation is a secondary type of glaucoma, caused by an increase in trabecular outflow resistance associated to trabecular amyloid deposition, with amyloid fibrillar aggregates in intertrabecular spaces of corneoscleral and uveoscleral meshworks and degeneration of endothelium cells of trabecular meshwork.

Experiences and lessons

Rarely, amyloid deposition in anterior segment can be much more exuberant than vitreous deposition. This asymmetry supports a significant role of the ciliary pigmented epithelium in the intraocular amyloid synthesis in these cases. Pupillary amyloid deposition and scalloped pupils have a stronger correlation to glaucoma than other ocular manifestations. Rarely, FAP-related glaucoma can be stable and well controlled by medical treatment alone.

Peer-review

This case is very rare and an interesting case.

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